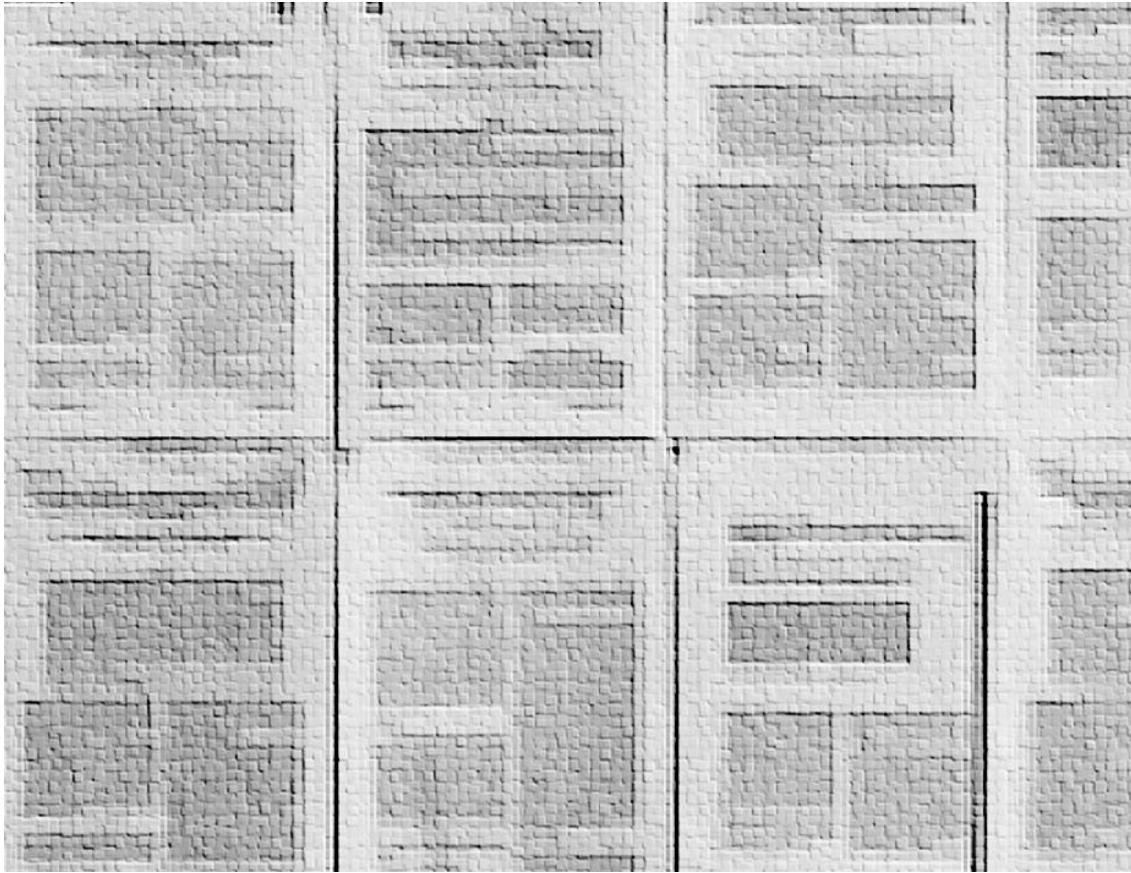


# Effects of BCG-treatment on Urinary bladder cancer, with focus on nitric oxide



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Institutet**

From MMK (Molecular medicine and Surgery)  
Karolinska Institutet, Stockholm, Sweden

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**Karolinska  
Institutet**

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# Effects of BCG-treatment on Urinary bladder cancer, with focus on nitric oxide

THESIS FOR DOCTORAL DEGREE (Ph.D.)

The thesis will be defended at Birkeaulan, Karolinska Universitetssjukhuset Huddinge

Friday, June 14, 2019 at 9:00 a.m.

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*Vissa dagar bär, man lyfter,  
svävar omkring med sin lycka.  
Färgerna vilar lättjefullt  
på sina föremål.  
Ljuden skojar vänligt med öronen.  
Fötterna är varma, huvudet svalt.  
Tankarna åker skridskor på blankis.  
De genomför alla tänkbara figurer  
utan att förlora balansen.  
Man kan sitta i lugn och ro,  
nysta ihop slingorna av sitt  
ihoprafsade liv.*

*Andra dagar,  
andra dagar ska vi inte tala om.*

**Tommy Olofsson "Om härliga dagar" 1976**

**To Fanny and Lova**



## ABSTRACT

Intravesical Bacillus Calmette-Guérin is used as a single agent or adjuvant therapy in the treatment of non-muscle invasive bladder cancer. In spite of being one of the most efficient immunological treatments in cancer, BCG for bladder cancer is afflicted with a number of unsolved problems. One third of the patients do not respond, and BCG treatment may delay alternative curative therapies. The exact mechanism of action of BCG on bladder cancer is still in question. Further research and investigation is required to understand how to identify the non-responders, how to optimize treatments and decrease side effects, and ultimately which patients are most suitable for treatment and will have the most favourable results.

In this thesis, we have examined the long-term effects and efficacy of BCG in bladder cancer, and in line with previous research in our group, investigated possible roles of nitric oxide in the BCG reaction. Our aim was to deepen the understanding of the BCG-reaction, and ultimately to identify a predictive marker for BCG-treatment of bladder cancer. We investigated the long-term efficacy of BCG at reducing recurrence, progression and cancer specific mortality (CSM); and more specifically if NO played a part in these processes

The third paper of this thesis showed that BCG reduced the long-term risk of recurrence (HR 0.40  $p < 0.0001$ ) and progression (HR 0.52  $p = 0.038$ ) in high-risk NMIBC in a fixed cohort followed for 15 years. Concerning progression, the risk reduction was only statistically significant in patients without concomitant CIS. For CSM there was no statistically significant risk reduction. The other two studies suggest a role for NO in BCG treatment of bladder cancer. Firstly, we have identified possible predictive markers in BCG treatment. In the same cohort as above, promoter and intragenic polymorphisms in NOS2 and NOS3 were associated with altered outcomes after BCG treatment. Secondly, we have confirmed earlier findings that NO concentration is increased in the bladder during the induction course of BCG treatment and that macrophages stimulated with BCG express both NOS2 and NO. We have shown *in vitro* that urothelial bladder cancer cells express NOS2 and NO and, perhaps more importantly that this reaction and BCG induced cytotoxicity depend upon activated macrophages. Interestingly, macrophage dependent BCG induced cytotoxicity was independent of nitric oxide.

Future implications of these findings require further research, most importantly to verify our findings about NOS polymorphisms and BCG efficacy in a larger cohort, but also to investigate the association between NOS polymorphisms and functional outcome (NOS expression and NO formation) in bladder cancer.



## LIST OF SCIENTIFIC PAPERS

- I. Thiel, T., Ryk, C., Chatzakos, V., Hallén Grufman, K., Bavand-Chobot, N., Flygare, J., Wiklund, P., de Verdier, P. Secondary stimulation from Bacillus Calmette-Guérin induced macrophages induce nitric oxide independent cell-death in bladder cancer cells. *Cancer Lett.* 2014 Jun 28; 348(1-2): 119-25
- II. Ryk, C., Renström-Koskela, L., Thiel, T., Wiklund, P., Steineck, G., Schumacher, M., de Verdier, P. Outcome after BCG treatment for urinary bladder cancer may be influenced by polymorphisms in the NOS2 and NOS3 genes. *Redox Biology*, 2015 Dec;6:272-7
- III. Thiel, T., Ryk, C., Renström-Koskela, L., Steineck, G., Schumacher, M., Wiklund, P., de Verdier, P. 2018. Intravesical BCG treatment causes a long-lasting reduction of recurrence and progression in patients with high-risk non-muscle-invasive bladder cancer. *World J Urol* 2019 Jan; 37(1):155-163

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## LIST OF ABBREVIATIONS

APC	Antigene Presenting Cell
BAK	BCG-Activated Killer cell
BCG	Bacillus Calmette-Guérin
BCL-2	B-Cell Lymphoma 2 protein
CD	Cluster of Differentiation
CIS	Cancer <i>In Situ</i>
CSM	Cancer Specific Mortality
CSS	Cancer Specific Survival
DMEM	Dulbeccos Modified Eagle's Medium
EDRF	Endothelial Derived Relaxing Factor
eNOS	Endothelial Nitric oxide Synthase
FAD	Flavin Adenine Dinucleotide
FBS	Fetal Bovine serum
FGFR2	Fibroblast Growth Factor Receptor 2
FMN	Flavin Adenine MonoNucleotide
GSTM1	Glutathione S-Transferase mu 1
HR	Hazard Ratio
IFN $\gamma$	InterFeron gamma
IL	InterLeukin
iNOS	Inducible Nitric Oxide Synthase
KO	Knock Out
LAK	Lymphokine Activated Killer cell
L-NMMA	NG-MonoMethyl L-Arginine
L-NAME	Nitro-L-Arginine Methyl Esther hydrochloride
LPS	LipoPolySaccharide
MBT-2	Mouse Bladder Tumor line 2
MHC	Major Histocompatibility Complex
MIBC	Muscle Invasive Bladder Cancer
NAT1	N-AcetylTransferase 2
NF $\kappa$ B	Nuclear Factor Kappa light cahin enhancer of B cells
NK	Natural Killer cell

nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NMIBC	Non Muscle Invasive Bladder Cancer
PARP	Poly ADP-Ribose Polymerase
PPD	Purified Protein Derivative
PTEN	Phosphate and Tensin Homolog
SNP	Single-Nucleotide Polymorphism
TGFβ	Transforming Growth Factor Beta
TH	T-Helper cell
TLR	Toll-Like Receptor
TNFα	Tumor Necrosis Factor Alfa
TRAIL	Tnf-Related Apoptosis Inducing Factor
TUR-BT	TransUrethral Resection of Bladder Tumor
UGT1a	UDP GlucuronylTransferase 1a

# **1 INTRODUCTION**

## **1.1 Urinary Bladder cancer**

### **1.1.1 Epidemiology**

In 2018 2800 persons in Sweden were diagnosed with bladder cancer, making it the 6<sup>th</sup> most common malignancy in total and 2<sup>nd</sup> to prostate cancer in the urinary tract. The prevalence of bladder cancer the same year was 24500 persons. The large difference between incidence and prevalence is due to the high proportion of low malignant superficial tumors that result in a high morbidity but low cancer-related mortality (CSM). The relative 5-year survival rate in 2015 was 70 %, and every year there are approximately 700 bladder cancer deaths in Sweden<sup>1</sup>. Incidence and mortality rates vary considerably by country with the highest rate recorded for men in Spain (age standardized incidence (ASI) 27/100000). Globally in 2018 bladder cancer was the 6<sup>th</sup> most common cancer among males with a ASI of 10/100000<sup>2</sup>.

### **1.1.2 Pathophysiology**

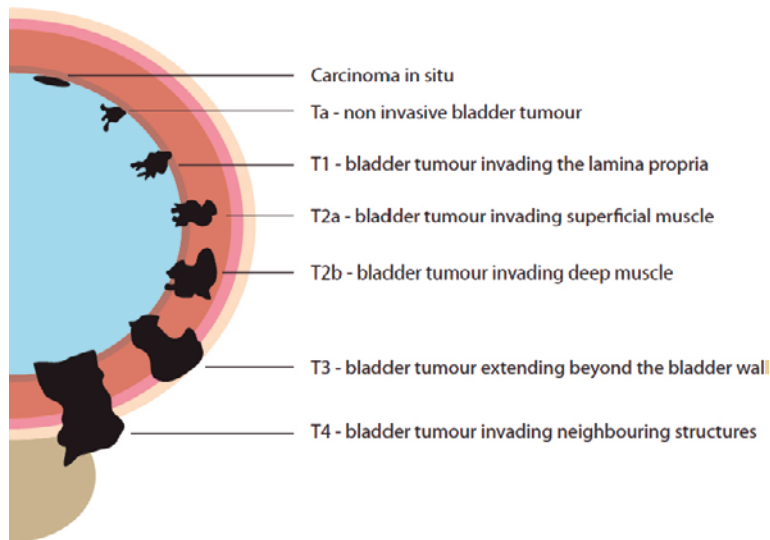
As in most cancers, the main risk factor for bladder cancer is age. Environment appears to contribute more than genetic factors, and smoking is estimated to contribute to one half of all bladder cancer cases<sup>3</sup>. The incidence differs between men and women (3:1). Chemical agents such as aromatic amines and polycyclic hydrocarbons are associated with bladder cancer, and 20 % of all bladder cancer is considered to be associated with exposure to carcinogens at work<sup>4</sup>. Chronic inflammation from urolithiasis, recurrent infection or an in-dwelling catheter is also considered to cause bladder cancer. Although not as important in bladder cancer as in other urological malignancies, hereditary factors do contribute to an increased risk. First grade relatives have a doubled risk and twin studies indicate that 1/3 of the risk of bladder cancer is due to hereditary factors<sup>5,6</sup>. Among genes identified, are FGFR3 (superficial tumors) and NAT2 and GSTM1 (resistance to tobacco smoke)<sup>7,8</sup>. Lynch syndrome is also associated with bladder cancer and is estimated to cause <5 % of all cases of bladder cancer<sup>9</sup>.

### **1.1.3 Histopathology and staging**

Histologically, urothelial carcinoma is the most common type of bladder cancer in Sweden, outnumbering adenocarcinoma and squamous cell carcinoma, by a rate of 19:1. The distinction between non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC) is important in urothelial carcinoma.

NMIBC is classified as Ta, T1 and cancer-in-situ (CIS). Pathological grade, according to the WHO grading system from 1973, ranges from G1 to G3. It is the most common type and accounts for 75 % of all newly diagnosed tumors<sup>10</sup>. Prognosis for NMIBC with a 5-year cancer specific survival (CSS) rate, ranging from >99%

(TaG1) to 70% (T1), is substantially better compared to MIBC, which have a 5-year CSS rate of less than 35 %<sup>11</sup>. Prognosis also differs in superficial cancer cases, with the risk of recurrence and progression depending on factors such as age, T-stage, malignancy grade, tumor size and number, and presence of concomitant CIS<sup>11,12</sup>. From these parameters, superficial tumors with a particular high risk of progression have been identified. High risk NMIBC consists of T1G1-T1G3, CIS and TaG3 tumors. T1 and CIS tumors are considered to be particularly malignant, with an almost 50% 5-year risk of progression<sup>13,14</sup>.



**Figure 1.** Stages in bladder cancer TNM classification.

#### 1.1.4 Treatment

Transurethral resection of bladder tumor (TUR-BT) is the initial standard procedure for urothelial carcinoma, being a mandatory diagnostic tool for all stages but also radical treatment for the majority of cases of NMIBC. Treatment options for muscle invasive tumors are radical cystectomy or radiotherapy with the addition of neoadjuvant or adjuvant systemic chemotherapy. In addition to TUR-BT, adjuvant treatment is also recommended in NMIBC. In low and intermediate risk NMIBC, mutamycin intravesical chemotherapy decreases the risk of recurrence<sup>10</sup>. In high risk NMIBC this treatment has no or little effect. The recommendation for these cancers is intravesical treatment with bacillus Calmette and Guerin (BCG), which is considered to decrease the risk for recurrence and delay progression<sup>10</sup>. The success rate of intravesical BCG as compared to other immunomodulating cancer treatment is high, with 2/3 of cases experiencing a 5-year recurrence-free interval<sup>15,16</sup>. For non- responders however, BCG treatment delays more radical treatment and procedures and thereby results in an increased risk of metastatic

disease and Cancer specific mortality (CSM)<sup>17</sup>. For NMIBC of particular high risk, such as extensive T1 with concomitant CIS, radical cystectomy without prior intravesical treatment is recommended.

## **1.2 BCG in urinary bladder cancer**

### **1.2.1 Background**

At the Pasteur institute in the 1910s, the researchers Albert Calmette and Camille Guérin developed the BCG vaccine from attenuated *Mycobacterium Bovis*<sup>18</sup>. In 1929, the American biostatistician Raymond Pearle, showed that cancer was less frequent in autopsied tuberculosis patients than in the population as a whole<sup>19</sup>. This discovery resulted in extensive research on BCG as cancer therapy. From animal studies in the 1960's and 70's, Zbar et al identified three prerequisites for BCG to be effective in cancer treatment; viable BCG, close contact between BCG and target cancer cells and an intact immune system<sup>20,21</sup>. The risk of adverse events and contagion made BCG difficult to use in most cancers. However the presence of tight junctions in the bladder mucosa made NMIBC a good candidate. Intravesical BCG as developed and introduced by Morales in the 1970's proved to be an efficacious and safe treatment for patients with NMIBC and is now an established standard treatment for high-risk superficial bladder tumors<sup>12,22</sup>.

### **1.2.2 BCG immunotherapy in bladder cancer**

#### *1.2.2.1 Treatment*

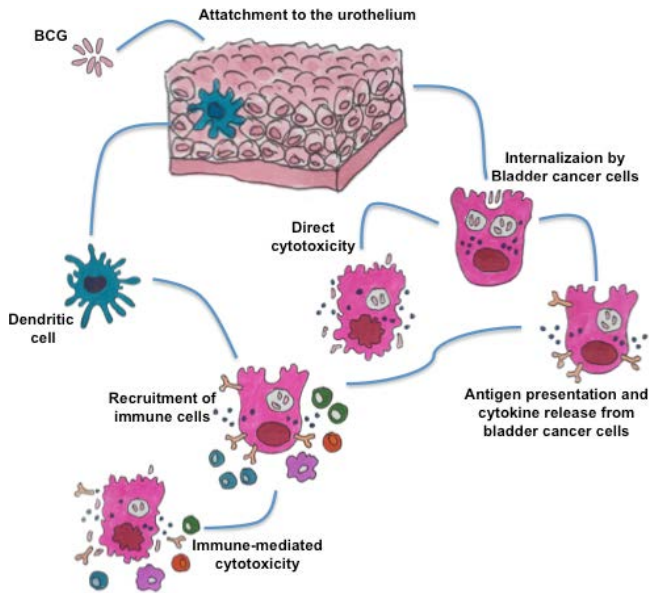
BCG is given either as monotherapy against CIS or as additional treatment after TUR-BT in exophytic T1- and Ta-tumors. It is recommended for all intermediate- and high-risk NMIBCs and consists of weekly instillations over a 6-week induction course. Thereafter, maintenance therapy is recommended with monthly instillations for 1 to 3 years, in cases of CIS and high-risk NMIBC<sup>12</sup>. Local adverse events such as bladder pain and dysuria are common (30 %), while severe adverse events such as reactive and systemic inflammation are rare (<5 %)<sup>23</sup>. While it is well established that intravesical BCG reduces the number of recurrent tumors in high-risk NMIBC, with a complete response rate as high as 80 % in primary CIS, there is uncertainty whether BCG-treatment prevents or delays muscle invasion, progression to metastases, or if it decreases cancer-specific mortality (CSM)<sup>24,25</sup>.

#### *1.2.2.2 Mechanism of action*

Despite extensive research, the exact mechanisms through which BCG mediates its anti-tumor effect remain unclear. The reaction is classified as a type 4 hypersensitivity reaction, and the response is thus mediated by cells rather than antibodies. The initial reaction is local and non-specific and is initiated by the binding of BCG to fibronectin and the uptake of the bacteria by tumor cells, urothelial cells



and anti-gene presenting cells (APC) in the bladder wall. The following reaction includes an activation of the innate and specific immune system as indicated both *in vitro* and *in vivo* by the increased concentration of cytokines and chemokines in the urine and the appearance of immune cells in the infected tissue<sup>26,27</sup>. Several lines of evidence suggest both direct effector-target cell contact and an active role of immune cells<sup>28</sup>.



**Figure 2.** Mechanism of action – BCG (Adopted from Redelman-Sidi 2014).

Natural killer cells (NK) are involved in the non-specific immune response against tumor cells. A subtype of these cells, BCG-activated killer cells (BAK), has been identified in the bladder wall of BCG-treated patients. In Knock Out (KO)-mice without BAK-cells the effect of BCG is reduced<sup>29</sup>.

Specific immune cells such as CD4-positive and CD8-positive lymphocytes are crucial for the effect of BCG. In the absence of these cell types, the anti-tumoral effect of BCG is substantially reduced<sup>30</sup>. The cytokine profile with a preponderance of interferon-gamma ( $\text{IFN}\gamma$ ), interleukin-2 (IL-2), IL-12 and IL-8 over IL-10 and IL-4 as well as the accumulation of cytotoxic T-cells, indicate a Th1 rather than a Th2 response<sup>31</sup>. In KO-mice without production of  $\text{IFN}\gamma$  or IL-12, the BCG reaction is absent<sup>32</sup>.

Mycobacteria such as BCG are intracellular microbes. Normally the internalization of mycobacteria is limited to APCs such as dendritic cells and macrophages. The

major histocompatibility complex (MHC)-mediated presentation of mycobacterial antigen on the cell surface results in an activation of a specific immune response involving both auxiliary and cytotoxic T-lymphocytes whose reactivity has been directed towards mycobacteria-infected cells. Activation leads to the elimination of both intracellular mycobacteria and of infected cells. Tumor cells seem to be more vulnerable to mycobacteria than normal urothelium. *In vitro* mutations in the phosphatase and tensin homolog (PTEN) gene and rat sarcoma gene (RAS) resulted in an increased uptake of mycobacteria in tumor cells and an increased expression of MHC II molecules on their surface<sup>28,33</sup>. Increased MHC II expression on tumor cells has been associated with a higher response rate in BCG treated patients<sup>34</sup>.

*In vitro*, BCG stimulation induces macrophage cytotoxicity against bladder cancer cells<sup>35-37</sup> and increased cytokines of macrophage origin have been detected in the urine of BCG stimulated patients, as well as macrophages within the bladder wall<sup>27,38</sup>.

Despite these findings, no *in vivo* studies have confirmed a positive role for macrophages in BCG treatment of bladder cancer. Instead, high numbers of tumor infiltrating macrophages (TAM) are associated with a reduced recurrence free survival interval after BCG treatment<sup>39</sup>.

#### 1.2.2.3 Predictive markers

Despite being the most effective intravesical therapy for NMIBC, recurrence and progression rates after intravesical BCG treatment range from 32-45 % and 10-13 % respectively<sup>12</sup>. Features such as tumor grade and stage, prior recurrence rate and tumor multiplicity are currently the best predictors for BCG response<sup>40</sup>. Aside from these there are no reliable predictive markers in clinical use for the early identification of non-responders to BCG-treatment. General tuberculosis immune status as indicated by a pre-BCG purified protein derivative (PPD) skin test, correlate with treatment outcomes as well as the level of urine cytokines IL-2 and IL-8 during treatment. However, interindividual variation and low specificity makes these less suitable as predictive markers<sup>41,42</sup>. The immunologic milieu surrounding the tumor environment such as the presence in the tumor of Th1 immune cells or NOS2 prior to BCG treatment has been associated with increased recurrence<sup>43,44</sup>. A Th2- polarized milieu nevertheless, with a high ratio of GATA-3+ and T-bet+ lymphocytes was found to correlate with a favorable response to BCG treatment<sup>44</sup>. The role of macrophages is complicated due to their dual roles in tumor immunological response, however, a high number of CD68+ tumor associated macrophages before BCG treatment, was associated with a higher recurrence rate after cessation of the treatment<sup>45</sup>. Attention has also been drawn to host related genetic factors and single nucleotide polymorphisms in IL-4, IL-6 and tumor necrosis factor alpha (TNF $\alpha$ ) genes have been associated with outcome after BCG treatment<sup>46</sup>. Epigenetic factors also appear to influence the post-BCG outcome. These include the methylation profiles of the genes coding for G/T binding protein (GTBP) and

thrombospondin (THBS), suppressor genes involved in DNA mismatch repair and neovascularization<sup>47</sup>. These findings are potentially significant and therefore warrant further research and evaluation.

## 1.3 Nitric oxide

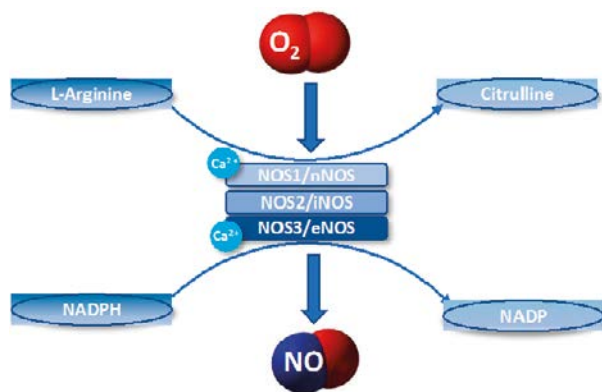
### 1.3.1 Background

Nitric oxide (NO) was identified as an important biologically active molecule in the 1980's and Ignarro, Furchgott and Murad were awarded the Nobel Prize in 1998 for its role in vasodilatation. Nitrite and nitrate formation by macrophages after BCG infection had also been observed by this time. Hibbs *et al* showed that formation of NO is associated with activation of macrophages while Moncada *et al* and Ignarro *et al* independently identified that endothelial derived relaxing factor (EDRF), the factor associated with vasodilation, was identical to NO<sup>48-50</sup>.

### 1.3.2 Formation

#### 1.3.2.1 Enzymes

The enzyme that catalyzes the reaction from which arginine is oxidized to citrulline and NO was identified in 1989 and named nitric oxide synthase (NOS). Three isoforms were determined, neuronal(n)NOS/NOS1, inducible(i)NOS/NOS2 and endothelial(e)NOS/NOS3. NOS1 and NOS3 are calcium dependent and produce NO transiently, at a low concentration, whereas activation of NOS2 leads to a sustainable production of NO at a high concentration independent of calcium. The co-factors in this reaction are nicotinic amide adenine dinucleotide phosphate (NADPH), calmodulin, tetrahydrobiopterin, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and heme<sup>51</sup>.



**Figure 3.** Nitric oxide formation by NOS enzymes.

### *1.3.2.2 Activation and regulation of NOS2*

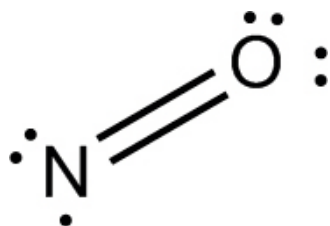
While NOS1 and NOS3 are constitutively expressed and regulated by calcium fluxes, NOS2 is very scarcely expressed in resting cells. NOS2 needs induction by an intrinsic or extrinsic factor to be formed and activated. Most of the active NOS2 is located in the cytosol or in the mitochondria<sup>52</sup>. The activity of NOS2 is both species- and cell dependent and the expression is regulated primarily by transcriptional and translational mechanisms. The pathways involved vary between cell types, but activation of the transcription factors NF $\kappa$ B and STAT1 seem to be essential for most cell types<sup>53</sup>. In murine studies, NOS2 expression is induced by cytokines and lipopolysaccharides (LPS). In humans, however, the mechanism is more complex and involves also epigenetic alterations such as methylation and histone modifications<sup>54</sup>. Mycobacteria induce NOS2 by activation of toll-like receptors in the macrophage cell wall and in mice this appears to be a mechanism for bacterial elimination<sup>55</sup>. In humans however, the regulation of NOS2 includes a posttranscriptional modification where mRNA is stabilized under the influence of micro (mi)RNA<sup>56</sup>. Transforming Growth factor beta (TGF $\beta$ ) is a decisive factor herein but also in translation and degradation of NOS2<sup>57</sup>. Function and formation of NOS2 depends on the micro milieu, in the modifying action by NO itself. In a feed-back mechanism NO inhibits transcription by S-Nitrosylation of factors such as NF $\kappa$ B, but also later on in translation by inhibiting NOS2 dimerization, and thereby transition into its active state<sup>58</sup>. Another factor related to the micro milieu is hypoxia. Stabilization of hypoxia inducible factor (HIF)-1 $\alpha$ , has been shown to be paralleled by an increased NOS2 activity in activated macrophages<sup>59</sup>. Substrate availability is also of importance. Arg-1 (arginase in cytosol), present both in macrophages and tumor cells, cleaves arginine into urea and ornithine, thereby causing a lack of substrate, which impedes the activity of NOS2. M2 macrophages, associated with low NOS2 activity are rich in arginase, a factor that might explain their immunomodulating features<sup>60</sup>.

## **1.3.3 Effects**

### *1.3.3.1 Chemical and physical properties*

NO is a small, uncharged molecule that, unlike almost all other molecules, contains an unpaired electron. This accounts for NO's chemical and physical properties and its ambiguous physiological and pathophysiological role in biology. Unpaired electrons are unstable and therefore all reactions that involve NO aim to stabilize the unpaired electron. This can happen in two ways; by pairing with another unpaired electron, or to share one electron with a transition metal. As a result, NO reacts either with other radicals, such as molecular oxygen (O<sub>2</sub>) or superoxide (O<sub>2</sub><sup>-</sup>), or with transition metals, most importantly iron (Fe) in the heme molecule. The former reaction ultimately results in oxidation of the nucleophilic components of nucleic and amino acids, in particular the thiols in cysteine and phenol rings in

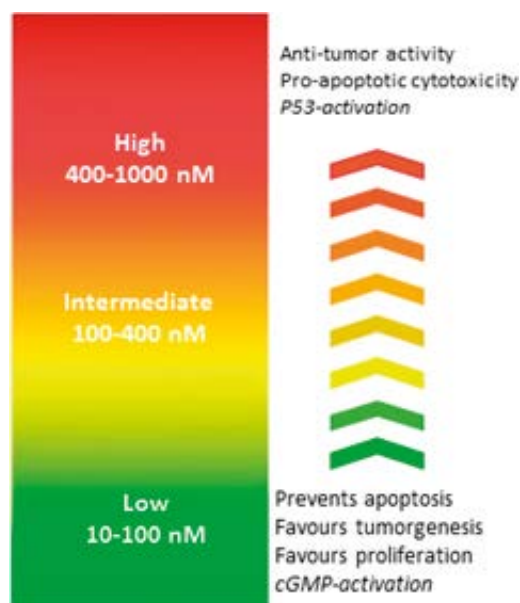
tyrosine and amines in DNA. This results in reconfiguration and altered function of proteins and nucleic acids containing these compounds. In the latter reaction where one metal ion, e.g.  $\text{Fe}^{2+}$ , turns into another, e.g.  $\text{Fe}^{3+}$ , the state of the metal-containing biologically active substance, such as heme in guanylate cyclase, is changed<sup>61</sup>. Unlike other known biological messengers, NO moves almost only by free diffusion<sup>62</sup>. This movement is not directional and is determined by concentration gradients. Being small and uncharged, NO has a high diffusion constant and diffuses from a higher to a lower concentration at an average speed of one cell length/ms (cell radius 4-15  $\mu\text{m}$ , total 150-300  $\mu\text{m}$ )<sup>62</sup>. As an almost non-polar molecule, NO is slightly soluble in water while it is highly soluble ( $\times 10$ ) in hydrophobic solvents<sup>62</sup>. As such, NO does not require specialized membrane carriers, easily crosses double-layered membranes and is not confined to the cellular compartment where it is produced<sup>63</sup>. The half-life of NO has been estimated to be 5-15 s<sup>62</sup>. Due to its high diffusion constant, and in spite of its relatively short half-life, NO acts rather in a paracrine rather than in an autocrine manner<sup>62,64</sup>.



**Figure 4.** Nitric oxide molecule with 1 unpaired electron.

### 1.3.3.2 Biological properties

The effect of NO is highly dependent on its concentration. At a high concentration, ( $\mu\text{M}$ ) following activation of NOS2, NO is cytotoxic, and is involved in the defense and deletion of pathogens and tumors<sup>56,65</sup>. At lower concentrations (nM) nitric oxide acts as a signaling molecule regulating smooth muscle relaxation and blood flow, neurotransmission and cell proliferation, survival and resistance. The biological effect at a particular concentration is not fixed, and depends also on the micromilieu and the affected cell type. Phosphorylation of soluble guanylyl cyclase (sGC) to cyclic guanosine monophosphate (cGMP), the reaction that results in smooth muscle relaxation and vasodilation, only requires some 10nM. At a concentration of 100nM, NO promotes angiogenesis and proliferation, while concentrations above 500nM result in formation of the highly reactive and cytotoxic compound peroxynitrite, which leads to reduced cell proliferation and cell death. The concentration of NO depends not only on the availability of substrates and the formation and activation of NOS, but also on the distance between the target and NO-forming cell and the rate of production and consumption of the molecule in vivo<sup>66,67</sup>.



**Figure 5.** *The concentration dependent effect of nitric oxide.*

In addition to concentration, the availability of gaseous oxygen and reactive oxygen species is decisive for the cytotoxic effect of NO. A high concentration of oxygen results in formation of  $\text{NO}_2$  and  $\text{NOOH}^\cdot$ , which are highly reactive and cytotoxic, whereas a lower concentration is associated with formation of  $\text{N}_2\text{O}_3$ . The latter is admittedly a weak oxidant, but still results in cellular damage by binding to amines and thiol groups, especially in a hypoxic environment<sup>68</sup>.

### 1.3.4 In apoptosis

Apoptosis is thought to be mediated by two pathways. Extrinsically, when FAS death receptors are activated by extracellular signals, or an intrinsically, starting in the mitochondria. Both of these pathways are affected by NO<sup>69</sup>.

The effects of NO on apoptosis seem to be dependent not only on the target cell type, but also upon the cellular location of NO and its concentration. Apart from direct damage to DNA, NO also targets a number of proteins involved in apoptosis. The effects herein are both pro- and anti-apoptotic and include the proteins caspases, p53, B-cell lymphoma protein 2 (BCL-2), poly ADP-ribose polymerase (PARP), cytochrome C oxidase and FAS death receptors. Cytochrome C oxidase is the final step of the electron transport chain of the mitochondria. NO competes at high concentrations with oxygen for its binding site and blocks Cytochrome C

oxidase activity when bound, resulting in impaired cell respiration and subsequent apoptosis. Other possible mechanisms of mitochondria-associated apoptosis are NO-derived activation of mitochondrial permeability transition (MPT) and oxidation of mitochondrial phospholipids, which leads to leakage of cytochrome C and activation of caspase cascades<sup>69</sup>. For PARP, an enzyme involved in both DNA repair and apoptosis, the picture is ambiguous, since NO, exerts both inhibition and activation depending on its concentration<sup>70</sup>. p53, “the gatekeeper protein”, that maintains genetic stability of the cell, and induces either cell arrest or apoptosis after DNA damage, is affected by nitrosative stress. *In vitro*, an accumulation of p53 has been observed in cells that were given NO-donors and in tumor cells that were transfected with NOS2. The complex role of p53 is enlightened by the fact that it by itself inhibits NOS2<sup>71</sup>. In an *in vivo* study, where rats were given thyroid tumors that had been either variably transfected with NOS2, those tumors with NOS2, showed a higher degree of apoptosis compared to those without NOS transfection. A high rate of apoptosis in combination with a low rate of transfected cells indicated that NO rather than the transfection itself was the cause of the apoptosis<sup>72</sup>.

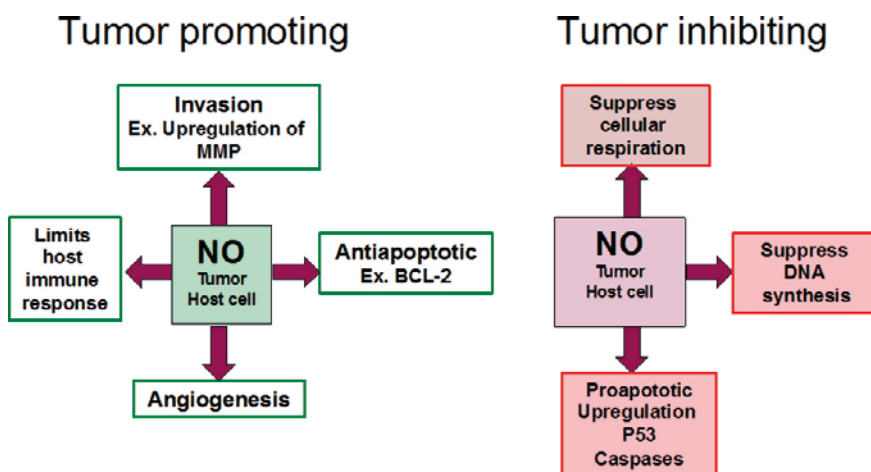
The proliferative impact of NO also includes anti apoptotic effects. S-nitrosylation prolongs the half-life of Bcl-2, an anti-apoptotic enzyme. Binding of NO to sGC and the activation of cGMP results in a reduced influx of calcium, which in turn is associated with reduction in apoptotic signaling. Another possible mechanism is NO-induced inactivation of caspases mediated by S-nitrosation<sup>70</sup>.

### **1.3.5 In cancer**

#### *1.3.5.1 General aspects*

There is multiple evidence that NO is involved in carcinogenesis<sup>73</sup>. By binding to metallo-complexes and sulfur residues in nucleic acids and proteins, NO causes DNA damage both directly from DNA strand breaks, and indirectly from inhibition and mutation of enzymes involved in DNA repair, such as PARP, and apoptosis, such as p53 and caspases<sup>73</sup>.

The concentration of NO and the duration of biological exposure are critical factors when analyzing the effects of NO in toto. In general, long-lasting low concentration of NO promotes tumor growth and progression while in comparison a substantially high concentration of NO, has a cytotoxic and proapoptotic effect, inhibiting tumor growth<sup>74</sup>. A long-lasting, low concentration of NO has been observed to promote epithelial mesenchymal transition, angiogenesis, invasiveness and metastasis of tumors, thus influencing almost all aspects of tumor progression<sup>66</sup>. The NO threshold for positive versus negative outcomes has not yet been identified, and indeed appears to differ depending on previous exposure. Acquired resistance to NO has been detected, and suggests that previous exposure to NO offers protection to a secondary higher dose exposure<sup>75</sup>.



**Figure 6.** The dual role of nitric oxide in cancer.

In the majority of published studies, an increased expression of NOS2 in primary tumor tissue is associated with a poorer prognosis. This association has been observed in bladder cancer, but also in other tumor types such as colorectal cancer, ventricular cancer<sup>43,76,77</sup>. In contrast, low expression of NOS2 was associated with progression and metastasis in pancreatic cancer studies done in mice<sup>78</sup>.

Tumor microenvironment is crucial to tumor growth, immune system evasion and progression. The interaction between tumor cells and surrounding cells such as fibroblasts, immunocytes and endothelial cells determines tumor evolution<sup>65</sup>. The presence of tumor-associated macrophages (TAM) has both positive and negative effects on overall tumor growth and outcome<sup>79</sup>. Depending on different environment stimuli, these cells appear polarized into two types; M1 macrophages that are cytotoxic and a major effector cell of the innate and specific immune response, and M2 macrophages that are wound healing and dampen immune response. M1 macrophages are thought to be induced by bacterial and tumor antigens, IFN $\gamma$  and TNF $\alpha$ , and are characterized by the expression of NOS2 and a high production of NO, whereas M2 macrophages are thought to be induced by parasites, IL-4 and IL-10. Interestingly, M2 macrophage activation is associated with a high expression of arginase, which (in theory) leads to a depletion of arginine in the cell and surrounding tissue and results in a reduced production of NO. It has thus been suggested that M2-macrophages contribute to a microenvironment that promotes tumor growth and immune evasion by an indirect lowering of NO, while M1 macrophages do the opposite<sup>60</sup>. The M1/M2 ratio seems to be important and may indicate a role for NO in tumor evolution generally. In colon cancer, tumors with a high number of macrophages expressing NOS2, were associated with better outcomes<sup>80</sup>.



#### 1.3.5.2 NO in bladder cancer

Evidence to support NOs importance in bladder cancer pathophysiology and treatment is scarce. Several studies, *in vitro* and *in vivo*, however, indicate that NO might have a role to play in the development of bladder cancer.

**NOS expression and NO formation in bladder cancer:** Bladder tumor cells are able to express NOS2 and form NO under certain circumstances. In the 1990's Konur *et al* showed that *in vitro* co-stimulation of bladder tumor cells with macrophages resulted in NO formation<sup>81</sup>. There are conflicting results whether NOS2 is normally present in unstimulated urothelium or not. In some bladder tumor tissue however, NOS2 is expressed at an increased rate, a fact that has been shown in several studies<sup>82,83</sup>. NOS2 is expressed in tumor cells and also by cells like macrophages in the surrounding stroma<sup>39</sup>. Increased amounts of NO in the bladder have been recorded among patients diagnosed with bladder cancer<sup>84</sup>, particularly those with CIS and the formation of NO may thus be dependent on the type of tumor<sup>85</sup>.

In summary, studies show that in some cases urothelial cancers express NOS2, a finding that was occasionally also associated with an increased concentration of NO in the bladder mucosa or urine of these patients.

**Negative and positive effects:** Like in other tumors, NO seems to have both negative and positive effects in bladder-cancer. Tumor cells are probably more sensitive to NO than normal urothelial cells. Normal urothelium cells were not affected when NO was given *in vitro*, however NO was cytotoxic to bladder cancer cells in a reaction that was reversible with NO-inhibitors<sup>86</sup>. In *in vitro* studies, Wang *et al* showed that the addition of NO was associated with a proliferation of lymphokine-activated killer-cells (LAK) involved in tumor eradication<sup>87</sup> and Tan *et al* found that bladder cancer cells that produced NO after NOS2 gene transfer, also increased apoptosis and expression of factor p53<sup>88</sup>.

NO-induced apoptosis, however, is of questionable importance in bladder cancer cases. The presence of arginase in the bladder mucosa of bladder cancer patients may indicate that the tumor cells are adapting to increased amounts of NO<sup>63</sup>. There is also evidence that NO is beneficial to the tumor. NOS3 and NOS2 have been associated with an increased angiogenesis in bladder cancer<sup>89</sup> and tumor expression of NOS2 among bladder cancer patients, was associated with a poorer prognosis<sup>90</sup>. In NMIBC patients, the presence of NOS2 in the tumor, has been associated with a higher recurrence rate<sup>43,91</sup>. In murine studies, expression of NOS2 in the tumor was associated with an increased metastasis risk, whereas mice that were given the NOS2-inhibitor L-NAME, had a decreased risk of metastasis<sup>90,92</sup>.

#### 1.3.5.3 BCG and NO in bladder cancer

Earlier studies in our group have shown increased levels of NO in the urinary bladder of BCG-treated patients and expression of NOS2 in the bladder mucosa of BCG-treated bladder cancer patients<sup>93</sup>. NO-concentration increased with an increased number of instillations, and NO levels stayed at increased levels for months after treatment completion<sup>94</sup>.

Macrophages and bladder mucosal urothelial cells *in vivo* form NO with BCG stimulation<sup>95</sup>. The exact role of NO in the BCG-reaction is unclear, but further studies suggest that NO may be a critical factor in the BCG-mediated anti-tumor effect.

NO mediates macrophage cytotoxicity in an arginine-dependent reaction<sup>49</sup>. A number of factors have been identified, *in vivo* and *in vitro* that trigger macrophages to form NO. These factors are both endogenous soluble proteins, such as the cytokines IFN $\gamma$  and TNF $\alpha$ , and exogenous, invasive pathogens such as *Mycobacterium bovis* and *E. coli*. The cytokines IFN $\gamma$  and TNF $\alpha$  are also involved in macrophage cytotoxicity. Cell to cell-contact is also critical for the cytotoxic effect of macrophages. Tumor type is another important factor.

The results of *in vitro* studies of the direct cytotoxic effect of BCG and NO on bladder tumor-cells have been contradictory. *In vitro* studies show that, T24 and MBT2 bladder tumor-cells express NOS2 and NO upon BCG stimulation, without adjuvant stimuli, and that this results in cell death. Further more, the formation of NO was reversible with NOS-inhibitors<sup>96</sup>. In direct contrast to these results, other research groups showed that macrophages and lymphocytes are necessary for BCG-induced NO-formation and cytotoxicity<sup>97,98</sup>. These latter studies are supported by the well-established assumption that an intact immune system is a prerequisite for the anti-tumoral activity of BCG<sup>20</sup>. Viability of BCG is also crucial<sup>21</sup> as high NO concentrations are toxic to BCG mycobacteria, a fact that may have clinical significance. In an *in vitro* study of BCG-stimulated macrophages, addition of NOS2-inhibitors promoted survival of intracellular BCG along with increased macrophage-derived cytotoxicity<sup>98</sup>.

Over all, there is uncertainty on the role of NO in the anti-tumor effect of BCG. Whether NO acts as a substantial tumor-inhibitor in BCG-treatment, or if it is just a marker for BCG-related immunological activity, is yet to be clarified.

#### 1.3.6 Influence of NOS polymorphisms

Polymorphism in biology is the presence of different variants or morphs, in a particular population of a single species. In medicine, polymorphism is used almost invariably for genes. Genetic polymorphism was defined in 1971 by Cavalli-Sforza

and Bodmer as “...the occurrence in the same population of two or more alleles at one locus, each with appreciable frequency, where the minimum frequency is typically taken as 1%”<sup>99</sup>. The most common existing variant of a gene in a population is named the wild type allele, while the others are called rare or variant alleles. The distribution of genes varies in different populations, and therefore the wild-type allele is population specific. Polymorphic studies of one group, may not be generalizable to other populations - a genotype that is beneficial in one environment for one disease burden, is not in another milieu, where group health faces another panorama of threats. Polymorphisms are present in every gene of an individual, and may be associated with vulnerability to infectious diseases and cancer<sup>100</sup>.

The first evidence of the importance of genetic polymorphisms in disease development was reported in the 1960's. A number of gene variants of erythrocytes were linked to susceptibility to malaria and explained the occurrence in certain areas and populations, of diseases such as sickle-cell anemia and thalassemia<sup>101</sup>. Another field of interest was cancer, and early works in the 1980, included studies on HLA polymorphism and leukemia, and CYP polymorphism and lung cancer<sup>102</sup>.

There are only a few familial cancers that are caused by a single known mutation in a tumor suppressor gene. Most hereditary factors however are due to lower risk genetic polymorphisms, that affect the susceptibility for developing a cancer<sup>103</sup>. These genes might influence specific proteins such as enzymes involved in cell cycle control, DNA repair mechanisms and the metabolism of toxic substances that are in turn affected in cancer.

Heritable factors have been estimated to cause 1/3 of all bladder cancer cases<sup>6</sup>, however, familial cases are scarce and only a few syndromes, such as Lynch syndrome, have been identified. Therefore, lower risk genes and their polymorphism may play a greater role than previously thought. There has been a particular interest in the interplay between environmental exposure and certain polymorphisms of enzymes involved in bladder carcinogen metabolism. Exposure of carcinogens such as aromatic amines have been linked to certain morphs of GSMT1 and UDP glucuronosyltransferase 1a gene (UGT1a)<sup>8</sup>. How these polymorphisms are linked to bladder tumor carcinogenesis is still poorly understood, although several genes involved in DNA repair and metabolism, have also been associated with p53 mutations<sup>5</sup>. Various morphs of genes that code for inflammatory mediators other than NO, have been associated with an increased risk of bladder cancer. Among them are genes coding for IL4 and IL6, the latter also associated with outcome after BCG treatment<sup>104,105</sup>. The results, however, are inconsistent and vary according to ethnic origin and carcinogenic exposure. Although there are many candidates, so far no gene polymorphisms are used as a predictive tool in BCG treatment of bladder cancer.

For NOS genes, there are a number of polymorphisms of carcinogenic importance that influence tumor progression and disease specific mortality.

Polymorphisms in the promoter region of NOS have been associated with greater risk and poorer prognosis in ventricular and lung cancer<sup>106,107</sup>. Intragenic single nucleotide polymorphisms (SNP) in the NOS gene are also of interest. While rs1799983 (Glu298Asp), an intragenic NOS3 gene SNP, has been associated with increased risk of ovarian cancer<sup>108</sup>, rs2297518(Ser608Leu), an intragenic NOS2 SNP, has been associated with gastric cancer<sup>109</sup>.

For bladder cancer, there are still only a few NOS gene polymorphisms associated with carcinogenesis and survival. In our group we have previously studied a number of genes. Variants of a microsatellite (CCTTT)<sub>n</sub> repeat polymorphism in the promoter region of the NOS2 gene were associated with an increased expression of NOS2<sup>110,111</sup>. People with these variants also had a lower incidence of bladder cancer. In bladder cancer patients however, the same variants were associated with an impaired prognosis and a reduced cancer specific survival<sup>112</sup>. For the NOS2 intragenic polymorphism rs2297518(Ser608Leu) there was a variant that was associated with an increased incidence of bladder cancer but a reduced risk of progression<sup>113</sup>. For the NOS3 promoter polymorphism rs2070744(-786T>C) there were variants associated with an increased risk for bladder cancer and for the NOS3 intragenic polymorphism rs1799983 (Glu298Asp) genotype was associated with grade<sup>114</sup>. Exactly how these polymorphisms of NOS2 and NOS3 influence the expression of NOS2 and NOS3, and the formation of NO, and other pathways of influence on bladder cancer, remain unknown.

## **2 AIMS OF THE STUDY**

1. To analyze whether TUR-BT with adjuvant intravesical BCG is superior to TUR-BT alone in patients with high-risk NMIBC.
2. To analyze whether nitric oxide synthase-polymorphisms, the NOS2-promoter microsatellite(CCTTT)<sub>n</sub>, and the NOS3 promoter polymorphism rs2070744 (-786T>C) and the NOS3 intragenic polymorphism rs1799983 (Glu298Asp), are possible markers of outcome after BCG-treatment for NMIBC.
3. To investigate the role of macrophage secreted factors (MSF) from BCG-stimulated macrophages on bladder cancer cells.
4. To analyze the influence of macrophage secreted factors on NO-production and cell death in bladder cancer cells.

## **3 MATERIALS AND METHODS**

### **3.1 Study populations and clinical assessment (Paper I–III)**

In Paper I, the study population consisted of 29 NMIBC patients planned for BCG treatment. In all patients, the risk of recurrence or progression was assessed as intermediate to high.

In Papers II and III the study populations consisted of a well-defined cohort of patients diagnosed with bladder cancer. Over a period of 2 years, from 1<sup>st</sup> of January 1995 to 31<sup>st</sup> of December 1996, all patients newly diagnosed with urothelial carcinoma (UC) in the Stockholm County were asked to participate in a prospective cohort study. In total 538 patients, 76% (538/705) of all patients in the county during that period agreed to participate. The entire cohort was described in a 5-year clinical follow-up in 2003<sup>11</sup> and in an additional article in 2010<sup>115</sup>. Parameters registered initially were; date of diagnosis, sex, age, stage, grade, tumor size, tumor multifocality, presence of concomitant CIS, and presence of detrusor muscle in the resected material. The same pathologist reviewed all histopathological assessments and the standard pathological report included data on stage, grade, presence of concomitant CIS, and detrusor muscle in the specimen. Histopathological classification followed the tumor, node, and metastasis (TNM) classification from 1978<sup>116</sup>. For tumor grading, the WHO 1999 malignancy grading system was applied<sup>117</sup>. High-risk NMIBC was defined as all non-muscle-invasive T1 and all G3 tumors, e.g., T1G1–G3, TaG3, and primary and secondary CIS, according to EAU standards<sup>118</sup> and previous results from our group<sup>11</sup>.

Patient records of all the original participants of Paper II and III were scrutinized in 2011. At that time, all surviving patients had been routinely followed up over a 15-year period. The other patients had died from either urothelial carcinoma or from other disease, as reported in the Swedish Cause of death Register. Follow-up time was defined as the time elapsed from date of diagnosis to death or last clinical evaluation. A number of parameters were collected from patient files in the follow-ups after 5 and 15 years. For our studies, we considered the following being most relevant; number and date of recurrence, and date of progression to muscle-invasive tumor, development of lymph-node and distant metastasis, type of therapy, and date and cause of death. For the polymorphism study, data on NOS2 and NOS3 genotype were also registered. Recurrence was defined as diagnosis of any new tumor in the bladder after the initial staging and grading by TUR-BT. Progression was defined as growth of the initial tumor into the muscular layer of the bladder or beyond, appearance of local and distant metastasis or death from bladder cancer. Cancer specific mortality was defined as death from bladder cancer as reported in the Swedish cause of death register. In the cases where progression had not been reported before Cancer specific mortality, patient files were checked for other

possible causes of death. In cases where cause of death was registered as bladder cancer without any previous progression, date of progression was considered as the same as date of death. All patients included in the 5-year clinical follow-up were also included in the 15-year follow up.

In Paper III, all 140 patients with high-risk NMIBC from this patient material were included. Of the 140 patients, 1 was excluded due to inconsistent data, 82 were treated with BCG and 57 were not. We genotyped 88 of these 140 patients using venous blood samples. Of these 88 patients 51 had received BCG-treatment and 37 had not. These patients were included in the in Paper II.

The polymorphisms investigated in Paper II; the NOS2 promoter microsatellite (CCTTT)<sub>n</sub> polymorphism, the NOS3 promoter polymorphism rs2070744 (-786T>C), and the NOS3 polymorphism rs1799983 (Glu298Asp), were chosen for the following reasons; all 3 had previously been associated with an altered production of NO and expression of NOS2 and NOS3 respectively<sup>110,119,120</sup> and all 3 had also been associated with the development and progression of bladder cancer<sup>112,114</sup>.

Informed written consent was obtained from all participants across all three studies. The studies were approved by the regional Ethical Committee.

### **3.2 Tissue collection (Paper II)**

Tumor tissue from all of the 538 participants of the study was collected during the initial TUR-BT in 1995 and 1996. Venous blood (normal tissue) from 359 of these participants was collected at a later time point and genotyped for the NOS2 (CCTTT)<sub>n</sub> promoter microsatellite polymorphism, the NOS3 promoter polymorphism rs2070744(-786T4C), and the NOS3 exon 7 polymorphism rs1799983(Glu298Asp). 88/359 of the participants of whom normal tissue was collected were included in the present study. These 88 participants were all patients with high-risk NMIBC.

### **3.3 Cell culture AND treatments (Paper I)**

Cell cultures of human bladder cancer cells (MBT2, T24 and J82) were maintained in RPMI supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (Invitrogen). In cell cultures of the monocyte/macrophage Raw 264.7, RPMI was replaced by Dulbeccos modified Eagle's medium (DMEM) and in addition to FBS and L-glutamine supplemented with 100 nM  $\beta$ -mercaptoethanol and 50  $\mu$ g/l of Pyruvate (Invitrogen). Cells were kept in cultures in a humidified incubator at 37 °C, 5% CO<sub>2</sub>, and plated the day before treatments.

### 3.3.1 Treatments

BCG (Medac) was reconstituted in FBS or DMEM, depending on cell type, and administered to tissue culture at a final concentration of either  $0,5 \times 10^6$  or  $5 \times 10^6$  CFU/ml. Lipopolysaccharides from *E-coli* (LPS, Sigma Aldrich) and IFN $\gamma$  (Sigma Aldrich) were added to the cell cultures as indicated, in order to provoke NOS expression. For experiments where MBT2 cells were treated with supernatant from RAW 264.7 macrophages, macrophages were treated one day before MBT2 cells. On the next day 40 % of the growth medium was replaced with centrifuged media from untreated and BCG-treated macrophages. N<sup>G</sup>-Nitro-L-arginine-methyl ester (L-NAME, Enzo Life Sciences), was used for inhibition of the enzymatic formation of NO.

For the *in vivo* detection of NO in NMIBC patients, the induction course comprised 6 once weekly intravesical instillations of BCG (BCG-Medac,  $2 \times 10^8$ - $3 \times 10^8$  colony forming units dissolved in 50 ml of saline solution). NO-values in patients were recorded, and patients with acute or chronic urinary tract infection (UTI), detected with urine test sticks (Multistix<sup>®</sup> 5) and confirmed with urine culture, were excluded from the study, as were patients with less than four registered NO-values. At the end 24/29 patients were included in the study results.

### 3.3.2 RNA extraction and cDNA synthesis

RNA isolation from cell lines was performed by using TRIzol-chloroform extraction. In order to lyse the cells and separate the RNA from other cellular components, attached cells were lysed in TRIzol (1 ml) and chlorophorm (200  $\mu$ l), shaken and incubated for 3 minutes, and centrifugated (12000 $\times$ g) for 15 minutes. The aqueous phase containing RNA, now separated from lipids and proteins, was then transferred to a new tube and mixed with isopropanol, incubated and centrifugated for 10 minutes, and the supernatant was removed. The remaining RNA pellet was then washed in 75 % ethanol, air dried, dissolved in DEPC water and stored at -70 °C. RNA integrity was confirmed by using a Bioanalyzer (Agilent Technologies). For real-time PCR, 2  $\mu$ g RNA was converted to cDNA by using the High Capacity cDNA reverse transcription kit (Applied Biosystems).

### 3.3.3 Real-time PCR

NOS2 mRNA along with the expression of 95 other genes (including expression of 8 reference genes) was measured by using a TaqMan<sup>®</sup> Array Mouse Immune Panel. Each array card contained four ports and was loaded with one untreated control sample and three treated samples. Real-time PCR was performed in an Applied Biosystems 7900HT system using default settings (Applied Biosystems).



Expression curves were manually re-analyzed and checked. Change in target-gene expression in relative to untreated control was calculated from the comparative Ct method using  $2^{-\Delta\Delta CT}$ . Reference genes were used to quantify the target genes.

### **3.3.4 Immunoblot analysis**

Protein expressed within the cultured cells, was extracted and separated from other cellular content, by lysing the cells in EBC lysis buffer (Tris, NaCl 120 mM, 0.5 % NP40, NF 100 nM, Sodium Orthovanadate 0.2 mM, PMSF 10 µg/ml, Apoprotinin 10 µg/ml, β-glycerophosphat 50 µg/ml)(Sigma Aldrich), and then centrifuged at 14000 rpm for 20 minutes at +4 °C. Bradford protein assay (Bio-Rad laboratories) was used to determine the protein content of the supernatant fluid. Electrophoresis, using a 4-8% SDS Pierce ProteinGel (Thermo Scientific) was used to separate the investigated proteins; NOS2, β-actin and PARP, from other proteins. The proteins were then transferred onto PVDF membranes using wet blot or an iBLOT, and then blocked with 0,5% BSA for one hour. Application of antibodies was performed in 2 steps, firstly with either a NOS2 antibody (BD-Biosciences), a β-actin antibody (Sigma Aldrich) or a PARP antibody (Cell Signaling) over night and then consecutively with HRP-conjugated secondary antibody. These secondary antibodies and a chemiluminescent substrate (Thermo Fisher Scientific) were used to detect the final protein signals on autoradiographs (Kodak X-O mat 2000 processor). Mouse macrophages and urothelial cancer cells stimulated with LPS and interferon-γ were used as positive controls for NO and NOS2 detection. All reagents were used according to the manufacturer's instructions.

### **3.3.5 Growth Experiments**

The presence and absence of viable BCG and bacterial contamination in growth experiments with macrophages (RAW) and bladder cancer cells (MBT2) were determined in the media from untreated and BCG-treated cell cultures. After centrifugation (14,000 rpm, 5 min) of the media, a fraction of the pellet or supernatant was plated on agar plates, dried, and then incubated at 37 °C in humidified plastic bags. Bacterial growth was checked weekly for eight weeks. The input fraction of BCG was used as a positive control and untreated supernatants and sterile filtered supernatants were used as negative controls.

### **3.3.6 Cell Cycle Analysis**

The DNA-content of every cell, as proportional to the phase in the cell cycle, was determined with flow cytometry (FACS) after staining DNA with propidium iodide using the CycleTest Plus DNA reagent kit (BD-biosciences).

In all experiments, kits were used according to the manufacturer's instructions.

### **3.4 NO determination (Paper I)**

#### **3.4.1 In cell lines**

Sealed tissue culture flasks were incubated at 37 °C for 4 hours, 25 ml of air was aspirated from the headspace of each culture flask using a syringe and infused into a chemiluminiscence NO analyzer (CLD 700, Eco Physics, Durmen, Switzerland) to measure peak levels of NO.

#### **3.4.2 In the human bladder**

Intramucosal NO concentration was measured using a previously described and validated method<sup>121</sup>. First a silicon catheter was introduced into the bladder. The catheter balloon was filled with 25 ml of room air via sterile syringe. After 5 minutes the air was re-aspirated into the same syringe and subsequently infused into a chemiluminiscence NO analyzer (CLD 700, Eco Physics, Durmen, Switzerland), where peak levels of NO were determined. Air from the examination room was collected concurrently with the procedure and analyzed for NO content. This background NO level was then subtracted from patient NO levels. The lower detection limit was 1 part per billion (ppb). Calibration of the analyzer was performed regularly using an electromagnetic controller with known concentrations of NO in N<sub>2</sub>.

### **3.5 Genotyping methods (Paper II)**

#### **3.5.1 Fragment analysis**

The NOS2 (CCTTT)<sub>n</sub> microsatellite promoter polymorphism was analyzed by DNA fragment analysis with a ABI Prism 3730<sup>®</sup> Genetic Analyzer (AppliedBiosystems). PCR primers, used for the amplification of analyzed gene sequence, were designed with Primer3 software and the forward primer was labelled with 6FAM<sup>™</sup>. PCR was performed using 13 µl reactions containing PCR buffer, MgCl<sub>2</sub>, dNTP, AmpliTaqGold DNA-polymerase (AppliedBiosystems) and primer according to the manufacturer's instructions. After 45 cycles of amplification, 1 µl of the PCR product was mixed with HiDi-formamide (AppliedBiosystems) and GeneScan-500 Liz Size Standard (AppliedBiosystems), heated for 3 min at 95 °C and cooled on ice. The sample was analyzed for DNA fragments using a ABI Prism 3730<sup>®</sup> Genetic Analyzer and data was analyzed with a ABI GeneMapper-v.4.0.

#### **3.5.2 Allelic discrimination**

The NOS3 polymorphisms rs2070744(-786T4C) and rs1799983(Glu298Asp) were analyzed by allelic discrimination on an ABI Prism<sup>®</sup> 7900HT sequence detection system (AppliedBiosystems). TaqMan primers and probes were purchased from AppliedBiosystems. PCR was performed using 5 µl with 10 ng DNA as a template.

The genotyping of amplified PCR products was scored by differences in VIC and FAM fluorescent levels in plate red operation on ABI Prism® 7900HT sequence detection system (AppliedBiosystems) using SDS-2.2.1. software.

### **3.5.3 DNA sequencing**

To verify authenticity of amplified sequences, 10% of the bladder cancer patients and population controls, were randomly chosen for DNA sequencing. ExoSAP-IT (GE Healthcare) treated PCR products together with a sequencing primer were added to sequencing reactions, performed with a BigDye® Terminator Cycle sequencing kit (AppliedBiosystems). After treatment with BigDye® Xterminator, reaction products were loaded onto an ABI prism 3720 Genetic analyzer (AppliedBiosystems). Analysis of the data was done by using Sequencing Analysis 5.2 software (AppliedBiosystems) and 4 peaks (<http://mekentosj.com/programs>).

## **3.6 Study design (Paper II)**

In Paper II, patients that were homozygous for a long set of repeats, ( $\geq 13$ ) of the NOS2(CCTTT)<sub>n</sub> microsatellite, were compared with patients who were not homozygous for a long set of repeats. For the NOS3 polymorphisms, due to the low frequencies of patients homozygous for the minor alleles (C in rs2070744, and T in rs1799983), these were grouped together with patients heterozygous for the 2 alleles (CT in rs2070744 and GT in rs1799983) and compared with the patients who were homozygous for the most common allele (TT in rs207074 and GG in rs207074). When performing the genotyping the researcher had no knowledge of treatment modality.

## **3.7 Statistical analysis (Paper I, II and III)**

Paper I: All assays were done with a minimum of 2 independent experiments. The results were expressed as the mean $\pm$ S.E.M. Differences between the groups were analyzed by ANOVA with the least significant difference as post hoc. The association between intravesical NO concentration and the number of BCG instillations was also analyzed with 1-way Anova trend analysis and polynomial regression. For statistical calculations SPSS statistics, version 20, was used.

Paper II: Patients with certain genotypes who received BCG were compared with patients who did not, in terms of recurrence, progression and CSM. Among patients who received BCG, patients with one particular genotype were compared with patients with other genotypes in terms of recurrence, progression and CSM. Univariable analysis of each of these terms was done for Kaplan- Meier survival estimates and curves using the Mantel-Cox log-rank test. Multivariable Cox proportional regression with stepwise selection was used to calculate hazard ratios

(HR), and to verify the prognostic significance of clinicopathological factors of significance (age, sex, stage and grade). Associations between the polymorphisms were calculated with linkage disequilibrium and Pearson's coefficient of correlation. HRs are shown with a 2-sided 95% confidence interval (CI). For statistical calculations SPSS statistics, version 21.0, was used.

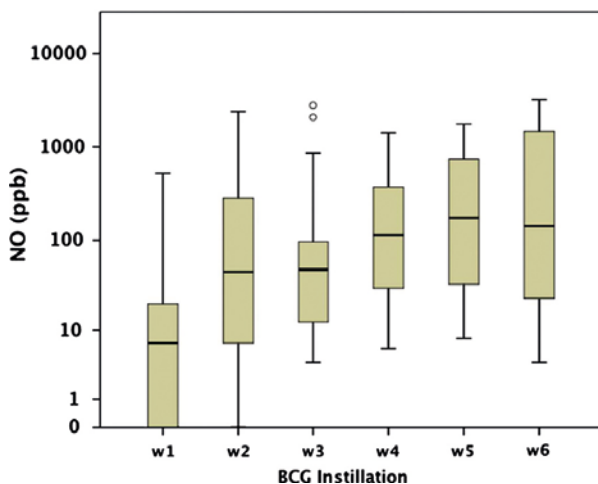
Paper III: Patients who received BCG were compared with patients who did not, in terms of recurrence, progression, and CSM. Univariable analysis of each of these terms was done for Kaplan-Meier survival estimates and curves using the Mantel-Cox log-rank test. Multivariable Cox proportional regression with stepwise selection was used to calculate hazard ratios (HR), and to verify the prognostic significance of clinicopathological factors of significance (age, sex, stage, grade, tumor size, multiple tumors, and concomitant CIS). In many studies, CIS is considered a separate entity. Separate analysis with and without CIS and concomitant CIS were, therefore, carried out in Paper III. HRs are shown with a 2-sided 95% confidence interval (CI). For statistical calculations SPSS statistics, version 21.0, was used.

In all of the 3 papers,  $p < 0.05$  was considered statistically significant.

## 4 RESULTS

### 4.1 Paper I

Our earlier observation, that NO is formed in the bladder of bladder cancer patients during BCG treatment, was confirmed in a larger cohort *in vivo*<sup>93</sup>. In the present study we discovered that the concentration of NO increases with an increasing number of instillations ( $p=0.001$ ), but with a substantial inter-individual variation (See Fig. 7).

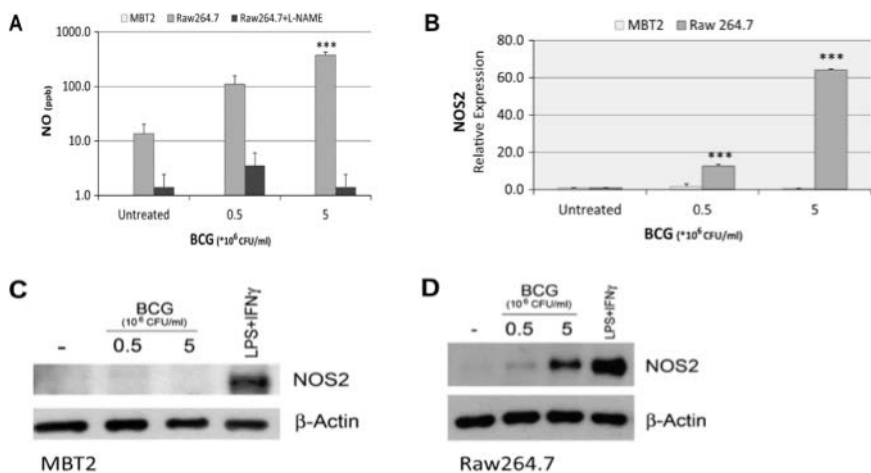


**Figure 7.** Intra-bladder nitric oxide concentrations after BCG-instillation displayed as a boxplot. The boxes represent IQR for each instillation, the band in each box the median value, whereas the whiskers are set at the highest and lowest (outliers excluded) values respectively. **BCG** *Bacillus Calmette-Guérin* **IQR** inter quartile range.

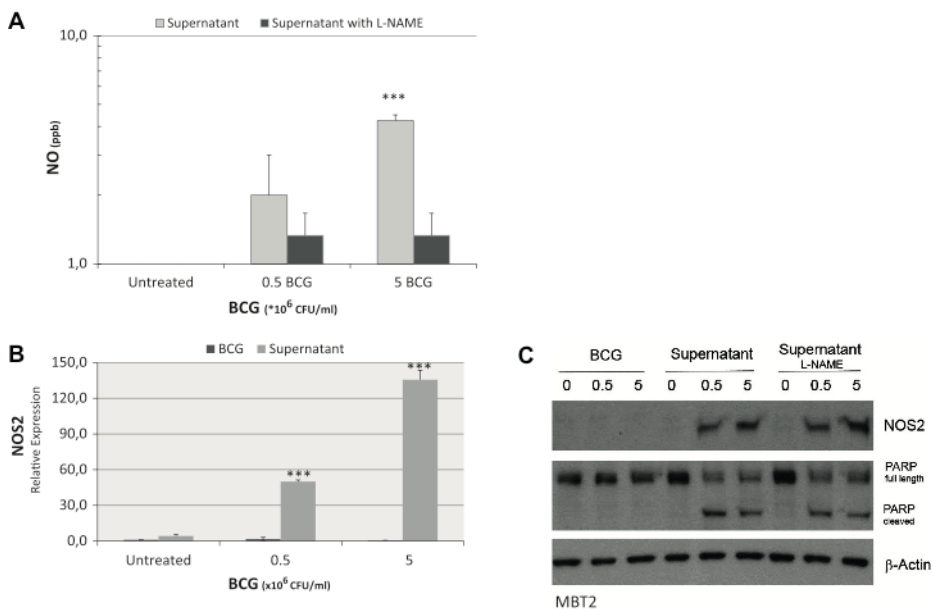
*In vitro* we discovered BCG to be cytotoxic to both murine bladder tumor cells (MBT2) and macrophages (RAW264.7). Cytotoxicity was dependent on macrophages. BCG induced apoptosis in MBT2 and RAW264.7 cells in a concentration and time dependent manner. This reaction was independent of NO and not affected by the addition of NOS inhibitors.

When RAW macrophages were stimulated with BCG, we also detected NO in a dose dependent release that corresponded to an increase in intracellular NOS2 mRNA and NOS2 protein expression. In contrast to RAW macrophages, stimulation of MBT2-cells with BCG did not result in detection of NO nor an increase in intracellular NOS2 mRNA or NOS2 protein expression in MBT2 cells (See Fig. 8)

When stimulated with BCG, RAW macrophages excreted a number of soluble factors. When the supernatant from these BCG-treated macrophages was added to the tissue culture media, a slight increase of NOS2 mRNA and NOS2 protein expression and NO formation was detected in the MBT2 cell culture (See Fig. 9)



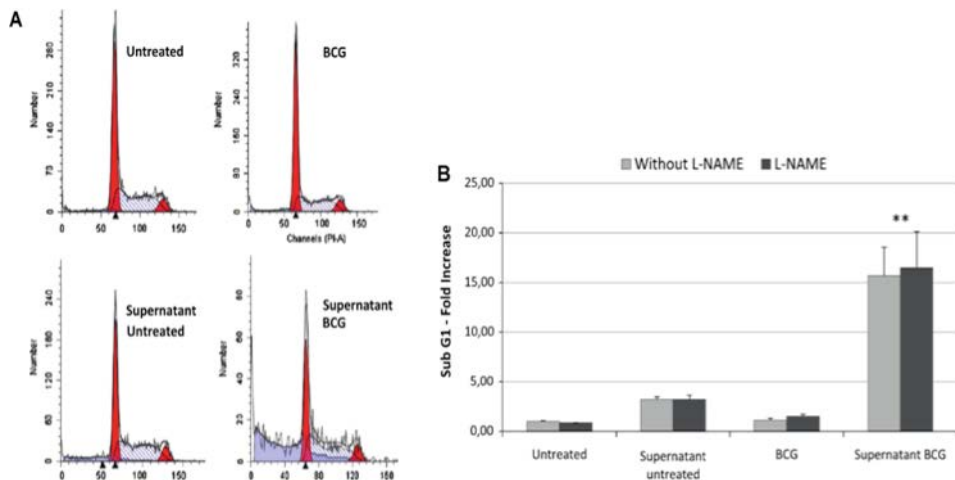
**Figure 8.** Nitric oxide formation (A) and NOS2 expression (B(mRNA) and C and D(protein)) in MBT2 cells and RAW264.7 according to treatment (No BCG,  $0.5 \times 10^6$  or  $5 \times 10^6$  BCG for 24 h). **BCG** *Bacillus Calmette-Guérin*.



**Figure 9.** Nitric oxide formation (A) and NOS2 expression (B(rtPCR) and C(western blot)) in MBT2 cells after adding supernatant from BCG treated macrophages (RAW264.7) to the culture medium. MBT2 cells were treated with supernatants from untreated RAW264.7 cells or cells treated with  $0.5 \times 10^6$  or  $5 \times 10^6$  BCG and/or L-NAME (100 $\mu$ l). **BCG** *Bacillus Calmette-Guérin*.

PARP cleavage, an indicator of apoptosis, was seen only in MBT2 cells treated with supernatant from BCG-stimulated macrophages. When L-NAME, a NOS2 inhibitor, was added to the macrophage culture media before BCG-treatment, apoptosis was not affected. (See Fig. 10)

Addition of supernatant from the non-BCG treated macrophage culture resulted in cell death among MBT-2 cells. The magnitude of cell death in the MBT2 cell culture increased, when BCG was administered to the macrophages. (See Fig. 10)



**Figure 10.** Cell cycle profiles after BCG-treatment. Propidium iodide stained MBT2 cells visualized by FACS analyses. (A) Cell cycle distribution among MBT2 cells according to treatment (no BCG, BCG, supernatant from untreated or supernatant from BCG-treated RAW 264.7 macrophages) (B) Cell death among MBT2 cells measured as the debris fraction in sub G1 and visualized as fold increase of fraction of cells in sub G1, according to treatment (as in (A)). *BCG Bacillus Calmette-Guérin*.

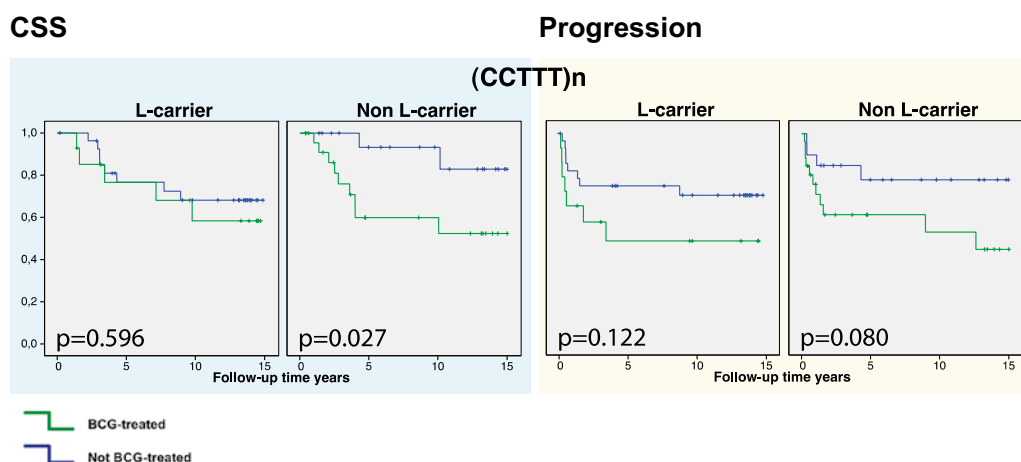
In an assay (TaqMan<sup>®</sup> Array Mouse Immune Panel) where expression of 95 inflammatory genes was measured in BCG-treated cells against an untreated control, BCG-treated macrophages had an increased expression of NOS2 and 26 additional genes. When tested in MBT2 cells immediately after BCG-treatment, only 3 of these additional genes (NOS2 not among them) showed increased expression.

## 4.2 Paper II

In this study we investigated the influence of previously identified NOS polymorphisms on stage progression and cancer specific survival after BCG treatment in patients with high risk NMIBC.

We investigated one NOS2 polymorphism and two NOS3 polymorphisms, of which all three influenced Cancer specific mortality (CSM) after BCG treatment, while the latter two also influenced outcome in terms of progression.

For the NOS2 (CCTTT)<sub>n</sub> microsatellite promoter (-2,6kb) polymorphism, genotyping was successful in all patients, of whom 51 had received BCG. BCG impact on CSM was associated with a short set of repeats (L<13). The risk of CSM in this group of patients (n=43) was significantly reduced for those who had received BCG compared to those who had not (HR: 0.12; CI: 0.02-0.98 p=0.048). Among patients with a long set of repeats (n=45) there was no statistically significant difference in CSM between those who had been treated with BCG and those who had not. (See Fig. 11)



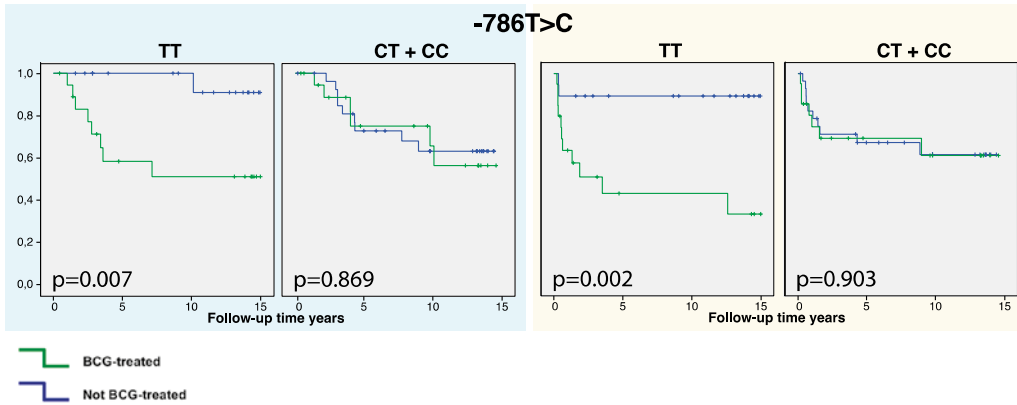
**Figure 11.** CSS and progression according to treatment (BCG or not) among patients with different variants of NOS2 (CCTTT)<sub>n</sub> microsatellite promoter polymorphism (L-carrier or Non L-carrier). **BCG** *Bacillus Calmette Guérin* **CSS** *Cancer Specific Survival*.

For the NOS3 promoter -786T<C (rs2070744) polymorphism, genotyping was successful in 87/88 patients, of whom 50 had received BCG. For this polymorphism, those with the TT genotype displayed reduced risk for CSM and disease progression after BCG, whilst patients with the CT/CC genotype did not benefit from BCG treatment in this regard. For the TT genotype, none of the patients who received BCG (n=21) experienced CSM compared to 7 out of 18 patients with who did not receive BCG. Also regarding progression, patients with the TT genotype had a reduced risk of progression after BCG-treatment (HR; 0.05; CI:0.01-0.42 p=0,005). (See Fig. 12)



## CSS

## Progression



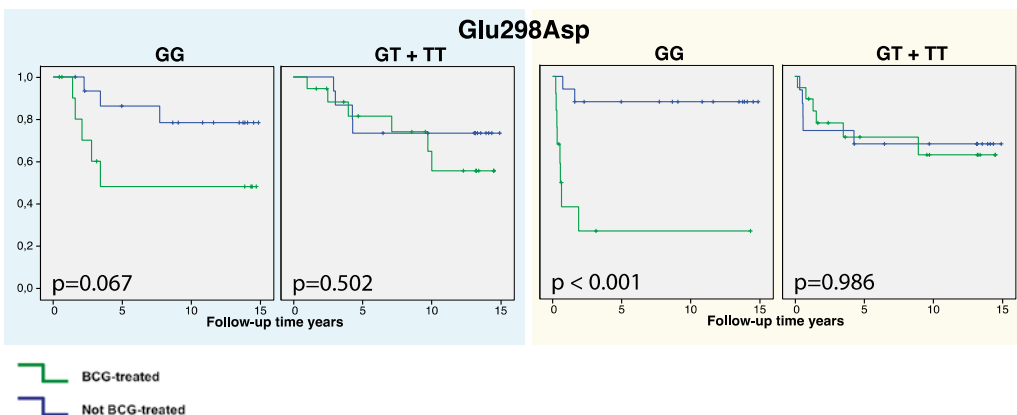
**Figure 12.** CSS and progression according to treatment (BCG or not) among patients with different variants of NOS3 promoter -786>C polymorphism (TT or CT+CC).

**BCG** Bacillus Calmette Guérin **CSS** Cancer Specific Survival.

For the NOS3 exon Glu298Asp (rs1799983) polymorphism, genotyping was successful in 64/88 patients, of whom 34 had received BCG. For this polymorphism, reduced risk for CSM and disease progression after BCG treatment were associated with the GG genotype, whereas patients with the GT/TT genotype did not benefit from BCG treatment in this regard. For the GG genotype, BCG treatment was associated with a reduced risk of CSM (HR: 0.16; CI: 0.03-0.84 p=0.030). Also regarding progression, patients with the GG genotype had a reduced risk of progression after BCG-treatment (HR; 0.10; CI:0.02-0.46 p=0.003). (See Fig. 13)

## CSS

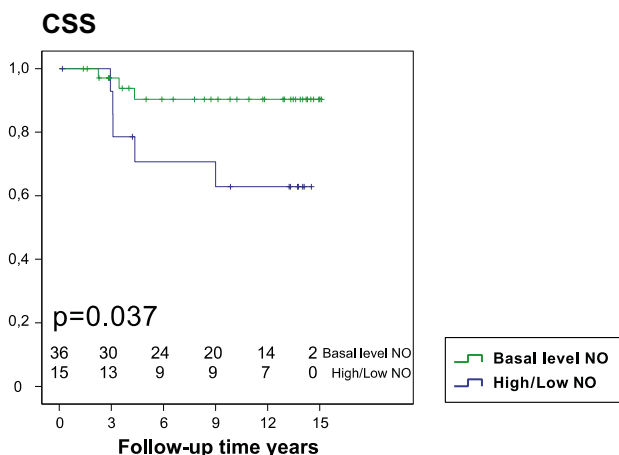
## Progression



**Figure 13.** CSS and progression according to treatment (BCG or not) among patients with different variants of the NOS3 exon Glu298As polymorphism (GG or GT+TT).

**BCG** Bacillus Calmette Guérin **CSS** Cancer Specific Survival

When the effects of these polymorphisms were combined in all patients who received BCG-treatment (n=51), patients with one or more of either NOS2 (CCTTT)non-L-carrier (homozygous), NOS3-rs2070744 TT or NOS3-rs1799983 GG genotypes, had a significantly better cancer specific survival (HR: 0.20; CI: 0.05-0.85 p=0.029) after BCG-treatment compared to those patients (n=15) who did not have any of these genotypes. In conclusion, those patients who had genotypes associated with basal levels of NO had benefit from BCG treatment as opposed to those who had genotypes that either increased or decreased the basal levels of NO (See figure 14).



**Figure 14.** CSS according to genotype (one or more of either NOS2 (CCTTT)non-L-carrier (homozygous), NOS3-rs2070744 TT or NOS3-rs1799983 GG genotypes, or not) among patients who received BCG treatment for bladder cancer. **BCG** *Bacillus Calmette Guérin* **CSS** Cancer Specific Survival.

### 4.3 Paper III

In this study we investigated the long-term effect of BCG treatment on recurrence, progression and cancer specific mortality.

139 patients with high risk NMIBC were identified and included. The majority of these patients, were high grade, size > 3 cm and staged as T1. BCG treated patients were younger (68.5 yrs. vs. 72 yrs.), of lower stage than T1 (68% vs. 93%), had more primary CIS (13% vs. 0%) and concomitant CIS (34% vs. 5%), and more G3 tumors (71% vs. 46%) than non-BCG treated patients. Detrusor muscle was obtained in as much as 83 % of the patients with T1 tumors, and similar in BCG treated and non-BCG treated patients (86 % vs 83 %).

Within 15 years of clinical follow up, 50 (36%) patients experienced stage progression and 38 (27%) patients had died of urothelial carcinoma. The majority of these patients progressed and died within 5 years from diagnosis. Over all, most of the events, occurred within 10 years of follow up after diagnosis. (Patients who died from urothelial cancer were older at first diagnosis and at progression) (See Table 1).

**Table 1. Clinical outcome n (%). BCG Bacillus Calmette-Guérin, T2NM stage 2–4, lymph-node metastasis or distant metastasis or CSM, CSM Cancer-specific Mortality**

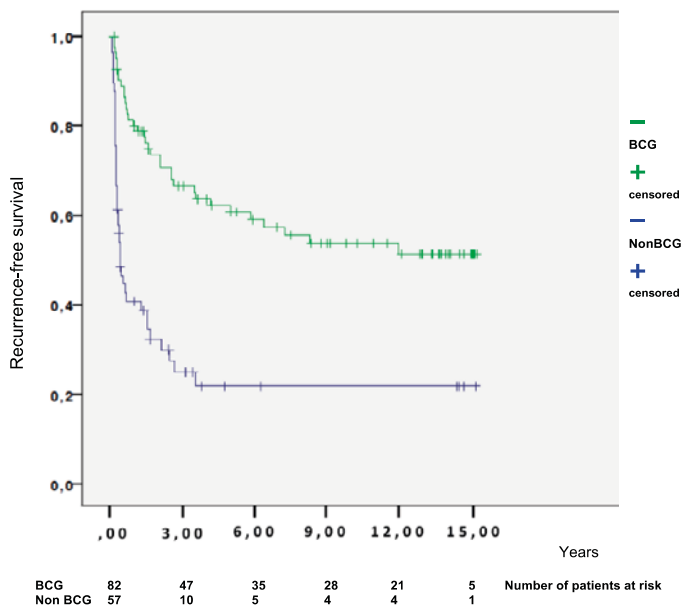
	BCG	Non-BCG	Total	<i>p</i>
Recurrence				
Yes	35 (42.7)	41 (71.9)	76 (54.7)	0.001
No	47 (57.3)	16 (28.1)	63 (45.3)	
Progression to T2NM				
Yes	21 (25.6)	29 (50.9)	50 (36.0)	0.002
No	61 (74.4)	28 (48.1)	89 (64.0)	
CSM				
Yes	16 (19.5)	22 (39.0)	38 (27.3)	0.01
No	66 (80.5)	35 (61.0)	101 (72.7)	
Overall death				
Yes	50 (61.0)	42 (73.7)	92 (66.2)	0.42
No	32 (39.0)	15 (26.3)	47 (33.8)	

*BCG* Bacillus Calmette–Guérin, *T2NM* stage 2–4, lymph-node metastasis or distant metastasis or CSM, *CSM* cancer-specific mortality

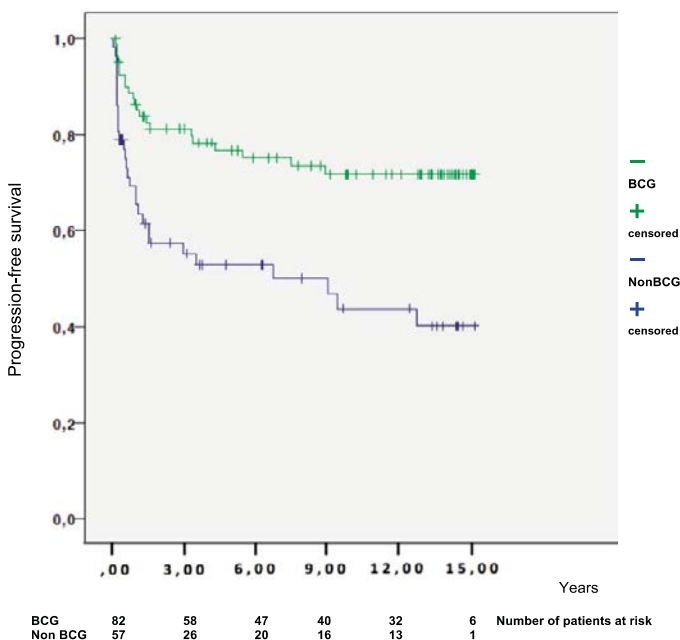
Alternative intravesical treatment was seldomly given and immediate cystectomy was performed in only 3 cases. When progression was diagnosed, cystectomy was the treatment of choice and performed in 25 patients. This treatment modality was more common in non-BCG treated patients as compared to BCG treated patients while the others were not.

Patients that received BCG-treatment had lower rates of recurrence than the group that did not receive BCG (hazard ratio (HR): 0.40  $p < 0.0001$ ). Also after exclusion of patients with primary CIS tumors ( $n=11$ ) there was a statistically significant difference between the 2 groups (HR: 0.40  $p < 0.0001$ ) (See figure 15).

For progression, the results showed a statistically significant difference between the BCG treated and the non-BCG treated group, both before (HR 0.52  $p=0.038$ ) and after exclusion of patients with primary and concomitant CIS (HR 0.41  $p=0.018$ ). However, after exclusion of primary CIS-patients, statistical significance fell below  $p < 0.05$  after adjustments for age and stage (HR 0.58  $p=0.075$ ) (See figure 16)

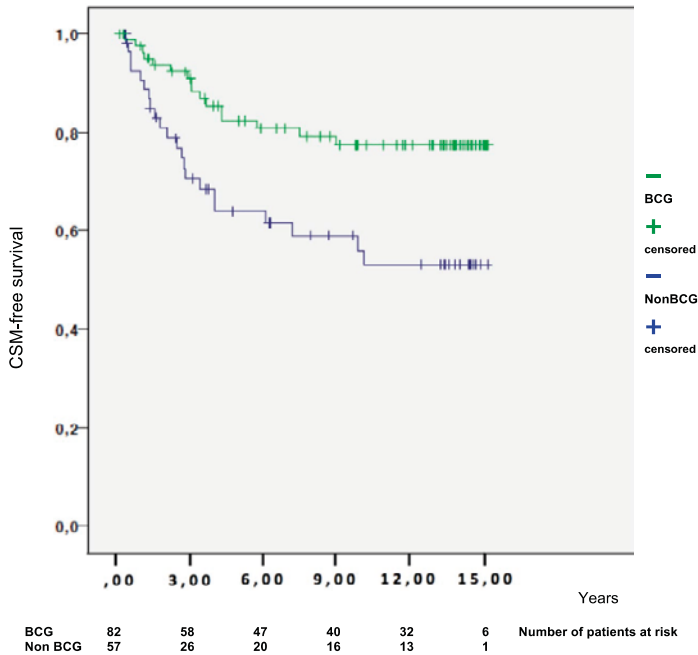


**Figure 15.** Recurrence-free survival according to treatment (BCG or not).  
**BCG** *Bacillus Calmette-Guerin*.



**Figure 16.** Progression-free survival according to treatment (BCG or not).  
**BCG** *Bacillus Calmette-Guerin*.

Death from urothelial carcinoma occurred at a lower rate in the BCG treated group as compared to the non-BCG treated group (20% vs 39%, HR 0.40 p=0.006 (see Table III)). However, after adjustments for age, stage, tumor size and number of tumors, no statistically significant difference could be measured (See figure 17).



**Figure 17.** CSM-free survival according to treatment (BCG or not).  
CSM Cancer Specific Mortality BCG Bacillus Calmette-Guerin.

## 5 DISCUSSION

The exact mechanism of action of BCG on bladder cancer is still in question. We need to understand how to identify non-responders, how to optimize treatments and decrease side effects, and ultimately which patients are most suitable for treatment and will have the most favourable results.

This thesis, examines the long-term effects and efficacy of BCG in bladder cancer, and in line with previous research in our group, investigates possible roles of nitric oxide in the BCG reaction. The aim was to deepen the understanding of the BCG-reaction, and ultimately to identify a predictive marker for BCG-treatment of bladder cancer. We investigated the long-term efficacy of BCG at reducing recurrence, progression and cancer specific mortality (CSM); and more specifically if NO played a part in these processes.

### 5.1 Efficacy of BCG

BCG was introduced by Morales et al in 1976 as a treatment against superficial, recurrent and residual bladder tumors. In this first study when BCG was given in 6 weekly instillations per urethram, only 1/9 patients experienced recurrent disease within the short follow-up (5 to 12 months). This study identified some of the by now well-known side effects of the treatment; dysuria, fever and malaise<sup>22</sup>. Morales observations on BCG regarding recurrence, were confirmed in several randomized trials and in 1990 BCG was approved by the Federal Drug Administration (FDA) for treatment of NMIBC. A large number of randomized studies, the majority of them comparing BCG with other intravesical treatments, e.g. mitomycin, interferon- $\alpha$  and epirubicin, showed that BCG reduces the number of recurrences after TUR-BT<sup>122</sup>. In order to improve the efficacy of BCG, maintenance treatment for 1-3 years was introduced and proven to be superior to an induction course alone<sup>118</sup>. Intravesical BCG-treatment is recommended as first line treatment in CIS and as adjuvant treatment in high-risk NMIBC by the EAU and the AUA<sup>12,123</sup>. In spite it being one of the most efficient immunological treatment for cancer, intravesical BCG for bladder cancer is afflicted with a number of unsolved problems. One third of the patients, and even more in the high-risk group, do not respond to BCG treatment<sup>16</sup>. Side effects are common and severe in comparison to other intravesical agents<sup>23</sup>. For the non-responders, aside from causing unnecessary side effects, BCG treatment may delay other potentially curative treatments, such as radical cystectomy or radiotherapy<sup>17,124</sup>. Since the introduction of BCG for bladder cancer, much effort has been done to improve its efficacy and to find predictive markers for recurrence and progression following BCG treatment.

As distinct from recurrence, there is still an uncertainty whether BCG reduces the risk of progression in NMIBC. In our present study we investigated the long-term effect of BCG on recurrence, progression and CSM, and in particular whether BCG treatment delays and prevents progression and CSM. In our study we showed that BCG reduce the rate of progression among patients with high risk NMIBC. This result is supported by a few relatively small, randomized studies from the late 1980's and early 1990's, of approximately 200 hundred patients with NMIBC, which showed that BCG reduced disease progression<sup>125,126</sup>. After the approval of BCG for NMIBC, randomized studies in which BCG-treatment was compared with no intravesical treatment at all have been difficult to perform for ethical reasons. Subsequent knowledge on the efficacy of BCG on progression was derived from prospective and retrospective cohort studies, or randomized studies in comparing BCG to other treatments. From these randomized studies there are conflicting results. In a meta-analysis from 2002, evaluating data from more than 4800 patients, Sylvester et al demonstrate that BCG therapy prevents, or at least delays, the risk of tumor progression<sup>25</sup>. When compared to mutamycin for NMIBC, BCG did not result in decreased progression or disease specific survival in an individual patient data meta-analysis from 2009 of 9 randomized trials including 2820 patients<sup>127</sup>. A recently published meta-analysis of randomized trials both on BCG vs. non-BCG and head-to-head trials on BCG vs. other intravesical agents (mutamycin, epirubicin, interferon) the authors conclude that BCG is superior to any other intravesical agent in decreasing progression<sup>122</sup>.

Non-randomized cohort studies, as our, reflect how BCG is used in practice but with confounding difficulties. As opposed to randomized studies patients tend not to be excluded due to age, gender, ethnicity or poor performance status. Many of these studies are characterized by heterogeneity of patient populations, a short follow-up period and a non-uniform definition of disease progression. In our present study a fixed cohort was followed over a comparatively long time for well-defined end-points. This study, among others shows positive progression effects from BCG treatment<sup>128</sup>.

Despite positive reduction effects in case progression, a well-defined cohort, a long follow-up and a high death rate, we could not show any statistically significant reduction of CSM in our study. Our finding is consistent with other research to date, as no randomized studies have shown a reduction of CSM in high-risk NMIBC after BCG treatment. In fact, there is scarce evidence that any of the intravesical agents currently used, BCG included, directly influence cancer specific survival (CSS) or overall survival (OS). One reason might be competing mortality among bladder cancer patients, who are at relatively high age at diagnosis, and have a higher comorbidity than expected<sup>129</sup>, probably due to common risk factors such as smoking. Prospective data in a number of studies show that OS is substantially lower in Ta-tumors and slightly lower in T1-tumors compared to CSS<sup>130</sup>.

The reasons for BCG failure may lie in clinical factors predictive for recurrence (tumor multiplicity and prior recurrence rate) and progression (tumor stage and grade) after BCG. Similarly, the very prerequisites for BCG-treatment (direct contact between target and BCG, viable BCG and an intact immune system) and the mechanisms of action that lie herein, may too hold the answers.

## **5.2 The role of NO and NO forming cells in BCG treatment**

Two of the studies underlying this thesis suggest a role for NO in BCG treatment of bladder cancer. Firstly, we have identified promoter and intragenic polymorphisms in NOS2 and NOS3 associated with outcomes after BCG treatment. Secondly, we have confirmed earlier findings that NO concentration is increased in the bladder during BCG treatment and that macrophages stimulated with BCG express NOS2 and NO. We have also been able to confirm that urothelial bladder cancer cells express NOS2 and NO. Unlike what has been shown in our earlier studies, expression of NOS2 and NO by bladder cancer cells depended on macrophage activation. The following sections will concern some aspects of these findings and possible future implications.

### **5.2.1 On the origin of NO**

From previous research in our and other research-groups we know that bladder cancer, and especially CIS, is associated with an increased formation of NO<sup>85</sup>. We have also detected a significant increase in NO concentration during BCG treatment. Despite being a highly diffusible molecule, the range of action of NO from its point of origin does not exceed more than the amplitude of 10 cells<sup>63</sup>. NO detected in the bladder during and after BCG treatment might thus originate either from the tumor cells, the macrophages and/or other immunocytes or the urothelial cells in the bladder mucosa.

Inflammatory pathways in BCG-patients are initiated by the activation of membrane-bound receptors. The membrane-bound receptors TLR2, 4 and 9 that recognize BCG are present in both macrophages and normal urothelium<sup>131</sup>. Binding to these receptors results in an activation of NFkB, and a subsequent production of NOS2 and NO<sup>55</sup>. Internalization of BCG is an important step for the macrophages to present or kill mycobacteria. Macrophages use NO in order to eliminate ingested mycobacteria<sup>89</sup>.

Urothelial cancer cells and normal urothelium are able to internalize BCG both *in vivo* and *in vitro*<sup>132</sup>. Based on a very limited number of studies there is some evidence that BCG-treatment results in NOS2-expression in urothelial cells *in vivo*. Immunohistochemical staining of the bladder mucosa showed that macrophages



and urothelial cells increased their formation of NOS2 after BCG treatment<sup>43,93</sup>. Immunohistochemical staining specifically for NOS2 in macrophages was only performed in one of these studies and indicated that NOS2 expression was higher in macrophages than in urothelial cells<sup>93</sup>. *In vitro*, there is until now only one study regarding NOS-expression by urothelial cells after BCG stimulation. In this study NOS expression was detected in primary culture of urothelial cells after the cell culture had been incubated with BCG<sup>94</sup>. Our study did not investigate NOS-expression in normal urothelium. Instead *in vitro* testing was conducted on immortally transformed urothelial cell lines. Most other *in vitro* studies of the impact of BCG on urothelium are performed in this way, and the results as compared to urothelial cells are equally difficult to interpret.

Bladder cancer tumor cells have the potential to express NOS and form NO. NOS expression in bladder tissue from bladder cancer patients has been detected in both bladder tumor cells<sup>91,133</sup> and in normal urothelium<sup>134</sup>. NO has also been detected at a higher concentration in the bladder of bladder cancer patients than in healthy controls. The increased formation correlated with the expression of NOS2<sup>84</sup>. Altogether, there is convincing evidence that bladder tumor cells are able to express NOS2 and form NO. For obvious reasons the influence of BCG on bladder cancer cells in humans *in vivo* has not been studied. In a few *in vitro* murine studies however, there is some evidence that tumor cells are able to form NO upon stimulation with BCG alone, which is in direct contradiction to our study results<sup>94,135</sup>. One possible explanation for the discrepancy may be that in these two studies another strain of BCG (Tice) was used than in our study (RIVM). After decades of passaging the now available BCG strains differ from a genetic point, a fact that may influence their virulence<sup>136</sup>.

Other cell types in the BCG reaction express and form NO upon activation. Upon *e-coli* infection of the mouse bladder, polymorphonuclear neutrophils (PMN) predominantly expresses NOS2<sup>137</sup>. PMNs have also been detected in the bladder wall after BCG instillation<sup>138</sup>. This cell type's importance in the BCG-reaction in bladder cancer is underlined by the observation in murine *in vivo* experiments that depletion of neutrophils revokes the antitumor efficacy of BCG<sup>139</sup>. There has been no investigation of neutrophil expression of NOS2 after BCG treatment, but based on studies of other microbials such as *E. coli*, the involvement of NO in microbial killing by neutrophils most probably is of very little importance. Neutrophils do not use NO for killing, neither for microbials nor tumor cells, and there is little evidence that depletion of NOS2 impairs neutrophilic tumoricidal efficacy<sup>140</sup>.

In summary, studies on tuberculosis have shown that macrophages are activated by mycobacteria and that macrophage action against BCG and other mycobacteria is mediated by nitric oxide. NOS2 has also been detected in macrophages in the bladder mucosa after BCG treatment. Our present study shows that macrophages express NOS2 and produce NO upon stimulation with BCG and that bladder cancer cell formation of NO is dependent on macrophage-derived soluble factors. In

contrast to what has been shown in urothelial cells, there is convincing evidence that macrophages produce NO upon stimulation with BCG, a fact that indicates that macrophages might be the most important source of NO in the bladder during BCG treatment.

### **5.2.2 On the role of NOS polymorphisms in NO production**

We hypothesized that NOS polymorphism would influence NOS activity and NO concentration in bladder cancer patients. Direct measurement of NO concentration and NOS activity was not performed in the patients in our study. We instead relied on several previous studies in which the current polymorphisms had been associated with altered enzyme activity. Polymorphisms in the promoter region of NOS2 ((CCTTT)<sub>n</sub>) and NOS3 (SNP -786T>C) had been associated with variations in expression of NOS2 and NOS3 respectively, as well as formation of NO<sup>110,111,120,141</sup>. Most of these studies concerned non-malignant diseases and until now there have been no specific studies on bladder cancer analysing the association between NOS polymorphism and NOS activity.

### **5.2.3 On the role of NO in BCG treatment and bladder cancer**

*In vitro* studies on the direct effects of BCG alone on cultured bladder cells have shown both cytotoxic and cytostatic effects of BCG<sup>36,142</sup>. In our study (Paper I) there was no indication that BCG alone exerts a cytotoxic effect on bladder cancer cells. *In vivo* the roll of direct killing by BCG is contradicted by the observation that the host has to have an intact immune system for BCG to work.

Therefore, most probably there are other factors that mediate the effect of BCG.

There are conflicting reports on the effect of NO on tumor cells. Whereas some studies show that NO enhances tumor growth and dissemination, others suggest a tumoricidal role for NO<sup>143-145</sup>. For bladder cancer several studies propose that some of the anti-tumor effect seen after BCG-treatment is actually mediated by NO<sup>86,96</sup>.

We have known for 30 years, at least *in vitro*, that NO is one of the effector molecules in macrophage-dependent tumor cytotoxicity. Already in 1989 Hibbs et al were able to show that NO caused the same pattern of cytotoxicity in hepatocytes as cytotoxic activated macrophages<sup>49</sup>. Although being an important player in the protection against mycobacteria, and despite convincing *in vitro* evidence, the specific role *in vivo* of macrophages and NO in BCG-treatment of bladder cancer has yet to be confirmed.

Mycobacteria, the genus to which BCG belongs, are aerobic intracellular bacilli. They are normally taken up by macrophages, where they multiply and prosper by utilising the cell's nutrients and evading being killed. BCG is an attenuated strain of *Mycobacterium bovis* and activates the immune system much the same way

as other mycobacteria. The immune response to BCG is both innate and adapted, the innate phase associated with activation of cell types such as granulocytes, monocytes and NK cells followed by a T-cell dependent adapted phase. The initial step involves activation of urothelial- and antigen-presenting cells, which produce chemokines (IL-8, MCP-1) and cytokines (IFN $\gamma$  and TNF $\alpha$ )<sup>146</sup> that then attract and activate neutrophils and macrophages. These cells reside in the bladder wall and give rise to the characteristic granulomas following intravesical BCG treatment. One feature of classically activated macrophages (M1) is an increased NOS2-dependent formation of NO<sup>147</sup>. In activated macrophages, NO and reactive nitrogen intermediates (RNI) contributes to the elimination of phagocytized mycobacteria<sup>148</sup>. Activated macrophages also act as antigen-presenting cells by presenting mycobacterial remnants in MHC2-complexes on their surface, thus activating T-cells in the adaptive immune response. A third role of activated macrophages is the formation of T-cell activating cytokines such as IL-2, IL-18 and IL-23<sup>97</sup>.

BCG-activated macrophages exert their cytotoxicity either by direct effector-target cell contact or by the release of soluble factors<sup>149</sup>. The knowledge about contact induced cytotoxicity is scarce but might include apoptosis-inducing factors such as Fas-ligands and Tnf-Related Apoptosis Inducing Factor (TRAIL). Soluble factors have been estimated to account for 50 % of the total killing, and involves TNF $\alpha$ , IFN $\gamma$  and NO<sup>150</sup>.

Despite the *in vitro* evidence supporting NO-involvement in mycobacteria-induced macrophage cytotoxicity, in our study BCG-induced cytotoxicity, exerted by soluble factors, did not involve NO. Our result is in line with the results of other studies<sup>98,151</sup>. In an *in vitro* study by Yamada et al from 2000, BCG-induced macrophage cytotoxicity against MBT-2 cells was enhanced rather than reduced when a NOS-inhibitor (L-NMMA) was added to the culture medium. The same study also indicated the importance of viable BCG, since cytotoxicity was only increased when living bacteria was used<sup>98</sup>. A reason for BCG failure might be that a high concentration of NO in the bladder kill viable BCG and thus decrease BCG mediated cytotoxicity.

The same study by Yamada et al, showed that BCG induced macrophage cytotoxicity was highly dependent on soluble factors such as IFN $\gamma$  and TNF $\alpha$ , cytokines that promote the cytotoxic activity of both the innate and the adaptive immune system. In our study we detected the expression in macrophages of a number of inflammatory genes in macrophages, among them IL6 and IL2, cytokines known to be involved in BCG induced cytotoxicity<sup>97</sup>. However, we did not detect expression of IFN $\gamma$  and TNF $\alpha$  genes in our study. In contrast to Yamada et al we did not perform analyses on the actual presence of cytokines in the supernatant. Time may be another influencing factor, as cytokines are expressed in a time dependent manner. Following the first BCG instillation, the first cytokines to appear in the

urine are IL1, IL6, IL8 and IL10. IFN $\gamma$  and TNF $\alpha$  have been detected only later during BCG treatment<sup>146</sup>. Yamada analyzed the supernatant from BCG stimulated macrophages after 36 hours, whereas our analyses were performed after 24 hours. This may explain why expression of these genes was not detected in our study.

The proximity of macrophages to their target might be an important factor *in vivo*. In CIS cases, macrophages existing in a high density close to the tumor cells are associated with reduced recurrence free survival time, whereas macrophages located beyond the lamina propria do not seem to affect this<sup>152</sup>. As NO is a molecule with a short half-life its effect decreases proportionate with distance. The concentration needed to exert cytotoxicity has been reported to be >200 nM<sup>153</sup>, which therefore requires close proximity between the macrophage and the target cell in order to exert an effect. Our previous studies showed that macrophages in the bladder wall express NOS2 upon stimulation with BCG<sup>93</sup>. We detected these activated macrophages in randomly taken biopsies of the bladder wall and still do not know whether they are also present in the peritumoral tissue, and thereby are able to exert cytotoxicity through the action of NO.

Comparative changes in NO concentration in the bladder before and during BCG treatment might reflect the distribution of different types of macrophages. Recent studies on the role of macrophages in bladder cancer have focused on tumor-associated macrophages (TAM), and as a pre-BCG predictive markers based on their presence and density<sup>154</sup>. Bladder tumors, as most solid tumors, have a high density of tumor-associated macrophages surrounding the tumor cells<sup>155</sup> and they appear in two phenotypes, M1 or M2. While the M1 phenotype is associated with tumor suppression and cytotoxicity, the M2 phenotype is associated with tumor progression and immune suppression<sup>60</sup>. The interplay between these macrophages and tumor cells is crucial for survival and dissemination of the tumor. The cancer cell has a number of strategies to evade immune destruction, among them the ability to recruit and polarize macrophages into the M2 phenotype<sup>156</sup>. In a number of studies, high density of TAM was associated with reduced recurrence- and progression free survival after BCG treatment<sup>39,157</sup>. This process includes an increased concentration of TGF $\beta$ . Among many effects, TGF $\beta$  down-regulates NF $\kappa$ B, a well-known transcription factor for NOS2, and thus transforms M1 into M2<sup>158</sup>.

An activated M1 macrophage produces NOS2 and NO when activated and TAM expression of NOS2 is used to identify polarized M1 macrophages. An increased formation of NO during BCG treatment may thus reflect a M1/M2 ratio that promotes tumor suppression and cytotoxicity, making NO an indicator of successful treatment rather than an effector molecule.

Most cell types are able to express NOS2 and NO with the right stimulation. In vitro, a mixture of LPS and IFN $\gamma$  evokes a cellular response characterized by the

formation of NO and a number of additional cytokines and chemokines<sup>86,159</sup>. As mentioned previously, there is both *in vitro* and *in vivo* evidence that BCG instillation leads to urothelial NOS2 expression and NO formation<sup>94</sup>. *In vivo* experiments on mice, show that in response to highly virulent invaders such as *e-coli*, urothelial cells are able to give rise to respond by producing NO and NOS2<sup>137</sup>. For less virulent bacterias, such as BCG, the reaction of the urothelium is probably less prominent. Whether NO formation and NOS expression in urothelial cells after BCG stimulation is an important effect *in vivo* in humans, however, still remains to be seen.

In summary, our study (Paper 1) suggests a role for macrophages in BCG induced cytotoxicity against bladder cancer cells. Based on *in vitro* and *in vivo* observations in other studies the increased NO formation during BCG treatment appears to have both negative and positive effects.

#### **5.2.4 On the role of NO in cancer and BCG failure**

NO has a dual role in carcinogenesis and tumor progression that depends not only on concentration and the quantity produced, but also on the characteristics of the cells and tissue where NO is produced<sup>92</sup>. The cells of the growing tumor have tumorigenic properties through the action of NOS2, whereas the cells of the surrounding stromal cells such as macrophages, have NOS2-dependent tumoricidal activity<sup>74</sup>. Increased NO production in the bladder before and during BCG treatment might thus have divergent effects.

NOS2 expression has been detected *in vivo* in a number of tumors. In a few of these studies expression was associated with improved prognosis<sup>160,161</sup>, but in the majority of the published papers NOS2 expression in different cancers was associated with impaired outcome in terms of recurrence, progression and CSM<sup>162-164</sup>. In one bladder cancer study, tumor expression of NOS2 was associated with decreased recurrence free survival<sup>134</sup>. In another study, all of the few patients who expressed intratumoral NOS2 experienced recurrence, as opposed to the patients who did not<sup>43</sup>.

In addition to our investigations, there are a several studies indicating a role for NOS2 polymorphisms in cancer development<sup>165-167</sup>. In the present study long repeats of (CCTTT)<sub>n</sub> of the NOS2-promoter satellite were associated with BCG-failure in high-risk bladder cancer cases. Earlier studies have identified polymorphisms associated with BCG-failure<sup>42</sup>, but so far there is only one other study that has studied BCG outcome related to NOS2 promoter polymorphisms and in this study no such association was seen<sup>168</sup>. However, this study differed from ours in regard to follow up (shorter), endpoint (recurrence only) and polymorphism (NOS2 -1026C/A).

There are conflicting results regarding the role of NOS3-polymorphisms in cancer development, with studies supporting both a tumor stimulating<sup>169,170</sup> and a tumor

suppressing effect of NOS3<sup>171,172</sup>. In our study the NOS3 genotype (c-allele of rs2070744) that presumably would result in a low expression of NOS3 and NO was associated with an increased cancer risk and BCG-failure. These results were supported by earlier observations in an *in vitro* study on a breast cancer cell line, MCF-7, which upon overexpression of NOS3 and NO was afflicted by increased apoptosis and impaired invasiveness. Another *in vivo* study on breast cancer patients had opposite results. In this study, the c-allele of rs2070744 was associated with less progression and improved survival. There was, however, no increased risk for breast cancer associated to the c-allele<sup>173</sup>.

In the famous article *Hallmarks of cancer*, from 2000 by Hanahan and Weinberg, a number of traits are listed that characterizes the transformation of a normal cell into a cancer cell. In the first update from 2011, another two traits (reprogramming of energy metabolism, evading immune destruction) were added to the original six (sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis)<sup>174</sup>. Among these traits there are several involving NO. *In vitro* cell culture experiments and murine *in vivo* studies have shown that an increased NO production by tumor cells results in decreased apoptosis, sustained angiogenesis<sup>145,175</sup>, increased tissue invasion and metastasis<sup>92</sup>, immune system evasion<sup>176,177</sup> and increased tumor growth and proliferation<sup>144</sup>. In several of these studies, NO inhibitors were able to antagonize this development.

In a study by Mitropoulos et al, patients with bladder tumors expressing NOS2 recurred after BCG, whereas the patients without intratumoral NOS2 did not<sup>43</sup>. In our study on polymorphisms the genotype associated with increased NOS2-expression was associated with BCG failure. Earlier discoveries on how tumors evade immune destruction may offer an explanation. Lymphocytes, crucial for BCG mechanism of action, are affected by elevated concentration of NO with decreased proliferation and cytotoxicity<sup>176</sup>. A high concentration as we have detected (Paper I) in the bladder during BCG treatment might impair lymphocyte function and decrease the effect of BCG. Another possible explanation is that nitric oxide locally produced by tumor cells, have a similar effect. Another finding is that continuous elevated concentration of NO makes tumor cells resistant to higher concentrations, as during the BCG reaction, and thus deleterious effects of NO<sup>178,179</sup>.

The dual role of NOS and NO in cancer may also depend on the cell type. In an murine *in vivo* study, inhibition of ovarian cancer cell proliferation was associated with activated NOS2 expressing macrophages<sup>180</sup>. Also endothelial cells seem to be of importance. In an *in vitro* study of malignant melanoma, endothelial cell derived NOS3-dependent NO formation had a cytotoxic effect on disseminating tumor cells<sup>181</sup>, a finding that might explain our result on BCG treatment and NOS3 polymorphism, where the genotype associated with low NO formation was associated progression and CSM.

Inhibition of NO in patients with NOS2 expression or a genotype associated with high NOS2-expression might improve outcome in bladder cancer after BCG-treatment. In fact, murine studies have indicated that orally ingested or intratumoral delivery of NOS-inhibitors had favourable effect on outcome in bladder cancer after BCG treatment<sup>92,151</sup>. So far NOS-inhibitors have not been used clinically in the treatment of bladder cancer or cancer in general. A clinical trial, where a NOS2 inhibitor was combined with docetaxel for advanced solid non-hematologic malignancies, showed no reduction of tumor growth compared to docetaxel alone, and increased side effects<sup>182</sup>.

Until recently there was no other immune-modulating therapy in bladder cancer than BCG. During the last few years, however, there has been a considerable development in cancer therapy targeting immunological mechanisms. In bladder cancer, immune checkpoint inhibitors have been approved for treatment of advanced MIBC with metastasis. In a recent study of NMIBC patients, PD-L1 was increased after BCG treatment in both tumor cells and tumor infiltrating inflammatory cells after BCG-treatment<sup>183</sup>. PD-L1-inhibitors have also been tested in combination with BCG with promising results in an orthotopic rat bladder cancer model<sup>184</sup>. Considering the suppressing effect of NO on immune cells, a combination of NOS2 inhibitors with immune checkpoint blockade may be an option in NOS2-expressing tumors. This combination is actually under current evaluation in patients with malignant melanoma in an on going study, however no results have been published yet<sup>182</sup>.

## 6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

From the studies of this thesis we concluded that BCG is an effective treatment in NMIBC regarding both recurrence and progression. However, recurrence- and progression rates (40 and 25 % respectively) were still considerable. Analysis of NOS2 and NOS3 polymorphisms might be a method to identify non-responders, and offer them alternative treatment at an early stage. The mechanism behind the NOS polymorphism influence on bladder cancer and BCG remains unclear, but might involve increased NOS2 activity and decreased NOS3 activity. Although there are a number of studies that suggest macrophage involvement in NOS-dependent cytotoxicity against cancer cells both *in vivo* and *in vitro*, our study did not support this hypothesis. However, our study suggests a roll for macrophages in BCG induced cytotoxicity against bladder cancer cells.

In comparing the cohort and randomized studies used in Papers II and III, several limitations have come to light. Specific data on BCG maintenance, rate of second look procedures, concurrent smoking status, and NOS2 and NOS3 protein and mRNA measurements in polymorphism, are lacking. On the other hand, recognized strengths in the studies were: the long follow-up, the fixed cohort and the low number of patients who were lost to follow-up, the quality of the initial TUR-BT (detrusor muscle in 83% of the T1 tumors) and the uniform pathologic assessment.

Planned prospective studies include detection of NOS expression in bladder tumors in NMIBC patients concurrently with NOS polymorphism analysis in order to detect the association between NOS expression, NOS polymorphism and BCG efficacy. For tumors with increased NOS2 expression, NO inhibitors that specifically target the tumor cells may be an efficient adjuvant to BCG. To my knowledge to date, there is no such pharmacological agent yet, and the development of homing NOS-inhibitors for use *in vivo* may be an interesting and efficacious area of future research. Prospective research could also focus on macrophage differentiation, the associations between NOS polymorphism and peritumoral M1 polarized macrophages and their subsequent impact on intravesical NO production, and outcomes after BCG treatment.



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